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PRINCIPAL INVESTIGATOR: Joanne S. Stolen

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43 DeNormandie Avenue
Fair Haven, New Jersey 07704-3303

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This publication consists of papers given at a workshop on Modulators of Fish Immune Responses held in Breckenridge, Colorado, USA, in September 1993. This is the first of a series of workshops to be held every two years to keep up with advances in the field.

The subject matter ranged from the use of fish as sensitive indicators of environmental contaminants to research, more basic in nature. Non-immunoglobulin-mediated ('innate') immunity was one of the key topics. Immediate results of xenobiotic influences and immunostimulators can be detected by assays such as phagocytosis, superoxide anion production, chemotaxis and serum lysozyme concentrations. Invertebrate (shellfish) phagocytes were also found to generate superoxide and process xenobiotics. Advances in cytokine studies in fish also bring intervention in fish immune systems within the realm of possibility.

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Volume one

Models for Environmental Toxicology Biomarkers Immunostimulators

Edited by **Joanne S. Stolen and Thelma C. Fletcher**

Modulators of Fish Immune Responses

Volume one

Models for Environmental Toxicology, Biomarkers, Immunostimulators

Edited by

Joanne S. Stolen
Thelma C. Fletcher

Section Editors

Douglas P. Anderson
Judith T. Zelikoff
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Stephen L Kaattari

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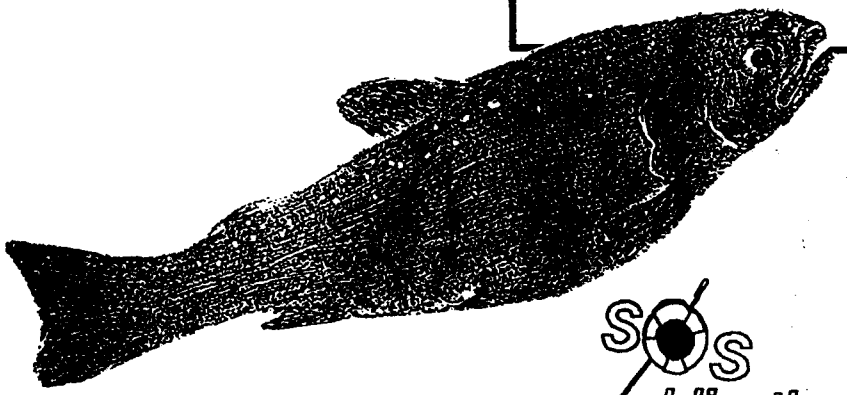

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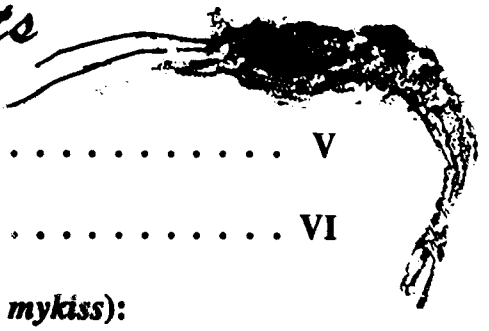

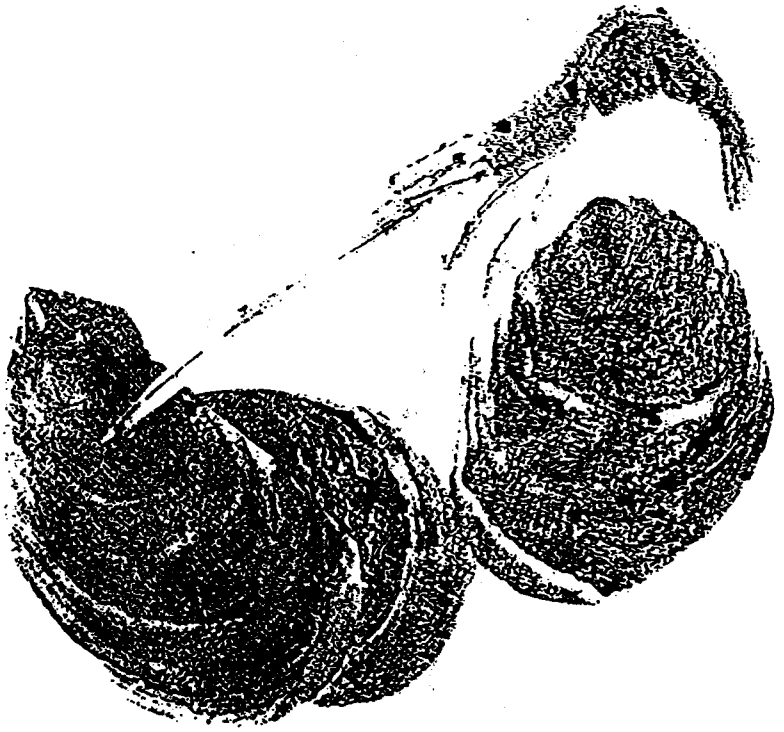

Preface

The Time Has Come

The time has come,
The fish immunologist said
To talk of many things.
Of lakes and rivers,
Of streams and springs,
Of oceans and of fishy things.
Of how we do modulate and stimulate,
And what pollution brings.

Joanne Stolen

In a world where pollution-caused immunosuppression is an increasing problem, **models for environmental toxicology, biomarkers and immunostimulators** are timely topics.

Because our feral fish populations have been decreasing rapidly due to pollution and loss of habitat, man is resorting more and more to culture to boost fish stocks. In aquaculture, disease is a major problem and the industry turns to immunologists for the production of vaccines and stimulation of immune responsiveness. We are also discovering that fish may be used effectively as models for hazard assessment for the aquatic environment.

This publication consists of papers given at a workshop on **Modulators of Fish Immune Responses** held in Breckenridge, Colorado, USA in September, 1993. This is the first of a series of workshops to be held every two years to keep up with advances in the field.

The subject matter ranged from the use of fish as sensitive indicators of environmental contaminants to research, more basic in nature. Non-immunoglobulin-mediated ('innate') immunity was one of the key topics. Immediate results of xenobiotic influences and immunostimulators can be detected by assays such as phagocytosis, superoxide anion production, chemotaxis and serum lysozyme concentrations. Invertebrate (shellfish) phagocytes were also found to generate superoxide and process xenobiotics. Advances in cytokine studies in fish also bring intervention in fish immune systems within the realm of possibility.

Chapter 1

Plasma Proteins of Rainbow Trout (*Oncorhynchus mykiss*): Immediate Responses to Acute Stress

Nora E. Demers and Christopher J. Bayne

Department of Zoology
Oregon State University
Corvallis, OR 97331-2914

ABSTRACT

Whereas chronic stress has been documented to have delayed immunosuppressive effects in a variety of vertebrates, the more immediate immunologic consequences of acute stressors are less known. We postulated that, as part of their 'fight or flight' response to alarm, rainbow trout would rapidly increase the efficacy of their natural immune system by means of increased concentrations of crucial plasma proteins. Plasma samples were taken from resting fish and from fish 5, 10 or 20 min after initiation of a stressful regime. Cortisol was significantly elevated in all stressed samples. Using crossed immunoelectrophoresis, it was possible to consistently identify, in plasma samples, twelve of a larger number of immunoprecipitate peaks. Altered areas under these peaks in different samples represented altered antigen concentrations. Four plasma antigens increased in concentration within 5 min of initiation of stress; none decreased. These rapid kinetics preclude dependence on gene activation, the basis of the acute phase response. One antigen that increased was identified as the third component of complement. Potentiation of natural immunity, which can reasonably be expected to be selectively advantageous at a time of trauma, may be one result of the increased concentrations of specific plasma proteins reported here.

INTRODUCTION

In the immediate aftermath of trauma-associated injury, it would be selectively advantageous for an animal to marshall its natural internal defenses for optimum efficacy. This appears intuitively obvious based on the common knowledge that rapid sequels to tegumental injury include entry of microbes into and loss of blood from the body. Knowledge of any trauma-associated changes in blood proteins is essential for a full understanding of the homeostatic response capacity of an organism. Such understanding will help optimize both the delivery of human health care and the formulation of protocols in animal husbandry including fish aquaculture operations. We contend that the vertebrate repertoire of responses to stressors includes rapid increases in the quantities of specific plasma proteins, among which will be some that serve the natural immune system. Precedent for such up-regulation of defenses is seen in similar changes accompanying exercise in physically fit individuals (Ortega *et al.*, 1993).

Contrary to this postulate, it has become widely accepted that stress is immunosuppressive. Agents of stress-induced immunosuppression are known to include corticosteroids (Ellsaesser and Clem, 1986; Mazur and Iwama, 1993; Wise *et al.*, 1993). Such negative changes in fitness, however, require hours to days to be manifested. Our contention that stress can induce positive changes addresses the kinetics of the putative response; the immediacy of need for heightened responsiveness following wounding (for example in the clotting and phagocytic arms of defense) should have resulted in the evolution of mechanisms for rapid changes in the levels of relevant plasma proteins. We envisage that such changes occur more rapidly than the so-called acute phase response, which requires gene activation and/or new protein synthesis. Increases in quantities of shock proteins following physiological insults, known as the acute phase response (Putnam, 1975, 1984; Koj, 1985; Schreiber, 1987; Baumann, 1988), result from gene activation and/or *de novo* protein synthesis. Our postulate of changes that occur within just a few minutes assumes the presence of presynthesized plasma proteins on reserve in the intact animal. As the source of most plasma proteins is the parenchymal cells of the liver, we envisage these to be a major source of proteins that respond most quickly to stressors.

We have examined rainbow trout plasma for altered concentrations of cortisol, glucose and macromolecular antigens following acute stress. Cortisol and four of twelve protein antigens increased significantly within 5.5 minutes of the initiation of stress. At least one of the antigens is a component of the complement system.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*, Shasta strain of the Cape Cod stock), were fed Oregon Moist Pellet, and raised (by Dr. Richard Ewing, Oregon Department of Fish and Wildlife) in pathogen-free well water at 10 - 12°C. They were used as 1 - 2 year fish weighing 150 - 450 g.

Bleeding and stress regime

Blood was collected by syringe from the caudal infravertebral arch. Samples were taken between 0930 and 1230 hr. To obtain blood from a resting fish (n = 15), the fish was netted, stunned by a concussion and bled within 1 min of approach to the tank. No further samples from the tank were considered resting, until at least 48 hr had elapsed. Stressed fish were processed immediately following treatment. Each fish was held in air for 30 sec, then in shallow water at ambient temperature in a 20 L bucket. Ten fish were concussed and bled after 5 min in the bucket, 9 after 10 min, and 4 after 20 min. Samples were dispensed into heparinized tubes on ice. Plasma collected after centrifugation was stored as aliquots at -70°C.

Cortisol and glucose

Plasma levels were kindly determined in the laboratory of Dr. Carl Schreck (Oregon State University) by radioimmunoassay (cortisol: Redding *et al*, 1984) and the 0-toluidine method (glucose: Wedemeyer and Yasutake, 1977).

Crossed immunoelectrophoresis (CIEP)

Five samples of each group (4 for the 20 min group) were pooled for analysis. Two dimensional electrophoresis was performed in 1% agarose and Tris-Barbital buffer, pH 8.6 (Weeke, 1973; Baumann, 1988). Plasma components were first separated at 10V/cm for 2.5 hr. Second dimension electrophoresis into the gel containing antiserum at 11% was at 2 V/cm for 22 hr at 10°C. Immunoprecipitates ('peaks') were detected by Coomassie Brilliant Blue staining of the rinsed, dried gels.

Rabbit antiserum to trout plasma antigens

Six New Zealand rabbits were injected with trout plasma emulsified in Freund's Complete Adjuvant, then later in Incomplete Adjuvant according to standard procedures. Sera containing antibodies recognizing 12 or more peaks in CIEP were pooled (300 mL) and stored as 1 mL aliquots at -70°C for use in CIEP.

Data analysis

Individual immunoprecipitate peaks were identified and labelled on photographs of the gels. Peaks were identified on the basis of their shapes, sharpness and resolution, and positions relative to the origin and to other peaks. The majority, but not all peaks, were identifiable on this basis in most gels. Areas enclosed within peak precipitin lines and the line of fusion between 1st and 2nd dimension gels were measured on a video screen image of the original gel. Data were entered into a Java Image Analysis System and analyzed using Statgraphics. One way analysis of variance was used to analyze cortisol, glucose and peak area data for separate group means. A natural log transformation was required of the peak area data to achieve homogeneity of variance.

Antigen detection on nitrocellulose (NC)

Antigens electrophoretically separated in a single dimension in agarose were transferred to NC by mechanical blotting. After blocking the membrane with 3% gelatin-Tris buffered saline, monoclonal anti-trout C3 (HYB-433 a gift from L. Goering-Jensen, Copenhagen) was used as a probe followed by peroxidase-labelled anti-mouse-Ig and Bio-Rad's Color Development Reagent.

RESULTS

Cortisol and glucose blood levels

In plasma samples taken from resting fish, the mean concentration of cortisol was 4.03 ± 1.04 ng/mL. The mean concentration of glucose was 69.5 ± 4.29 mg/dL. Significant increases were detected for cortisol as early as the 5 min sample, and for glucose in the 10 min sample (Figure 1). Both continued to increase in concentration for the duration of the 20 min stress regime as follows: cortisol at 5 min 78.92 ± 14.03, at 10 min 119.08 ± 18.61, and at 20 min 165.66 ± 21.89 ng/mL. Glucose at 5 min was 79.7 ± 4.26, at 10 min 92.0 ± 4.15, and at 20 min 116.0 ± 5.03 mg/dL.

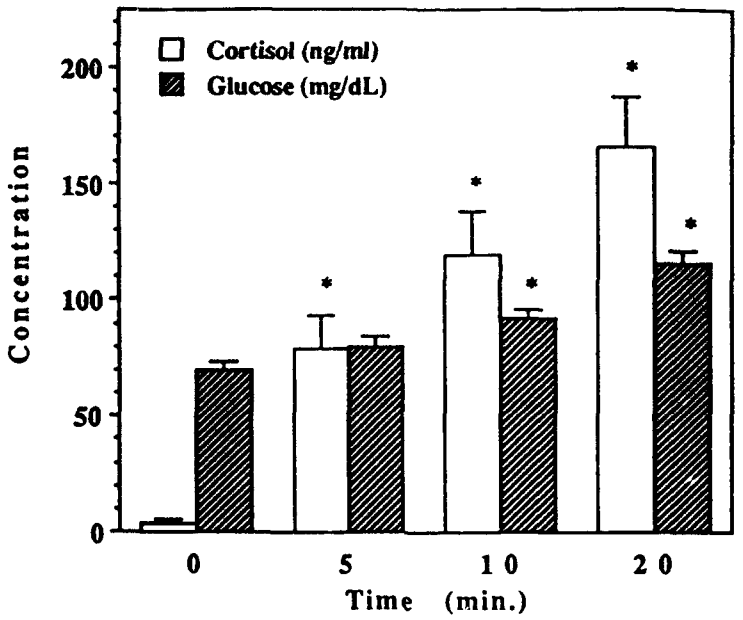

Figure 1. Concentrations of cortisol and glucose in plasma from rainbow trout at rest (n=15) and after 5 (n=10) , 10 (n=9) or 20 (n=4) min handling stress. Mean values ± SE. Values that were significantly different from control values ($p < 0.0001$) are indicated by *.

Plasma antigens detected with CIEP

When the same plasma was run in replicate, areas under the equivalent peaks in the different gels varied slightly. For this reason, and to conserve antiserum, plasma lots from different fish were pooled. Ten separate aliquots of the 'resting pool' were run on CIEP; the 5 min pool was run 8 times, the 10 min pool was run 7 times and the 20 min pool 8 times, although some peaks were not measured on every run.

Twelve peaks were consistently identifiable on most gels (Figure 2). When the mean values for areas under these peaks were compared, four (Peaks 3, 5, 9 and 12) were found to have increased significantly in stressed samples (Figure 3). Whereas peaks 9 and 12 increased continually over the 20 min, peaks 3 and 5 reached maxima in the 10 min samples, and declined thereafter. Other peaks either did not change significantly in area, or increased such that the antigen exceeded the precipitating capacity of the antibody in the gel and was not measurable.

When purified trout C3 (a gift of T. Yano and S. Kaattari) was added to plasma from a resting fish, peak 3 increased in area, identifying this as C3 (Figure 4). Peak 3 was similarly enlarged in stressed plasma. As the area under a peak is proportional to the antigen concentration, we ran C3 in CIEP and measured areas for peaks of known C3 concentration. Using the resulting correlation (Figure 5), we determined that C3 concentrations in plasma pools were as follows: 0.72 mg/mL in resting plasma, and 1.02, 1.20 and 0.95 mg/mL after 5, 10 and 20 min stress respectively. When purified C3 and appropriate unknowns were probed on NC using a specific antibody after single dimensional agarose electrophoresis (not shown), the locations of the purified C3 and of a strongly recognized antigen in salmonid plasma were identical. The electrophoretic mobility of this antigen relative to the start point was the same as that of peak 3 in CIEP, and is also consistent with that reported by Perrier and Perrier (1984).

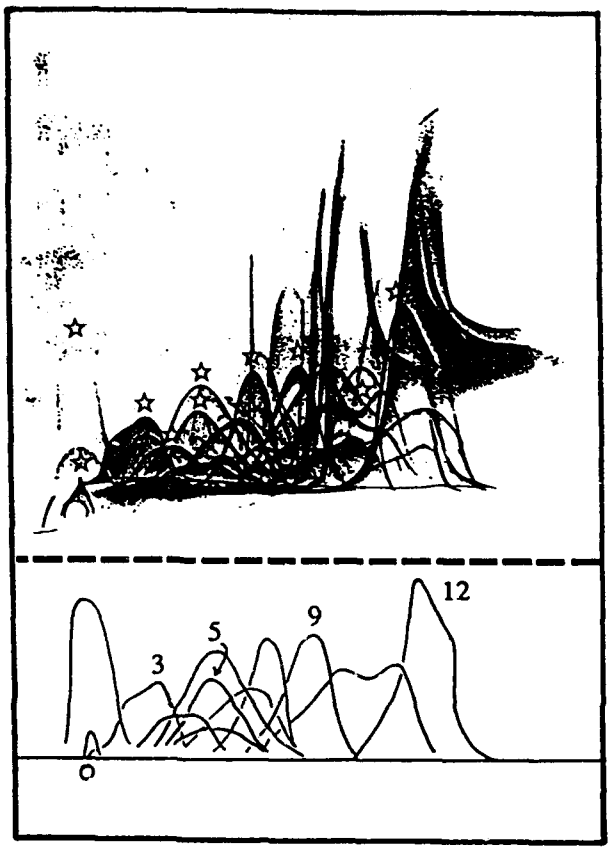

Figure 2. Plasma proteins of rainbow trout immunoprecipitated by rabbit anti-trout plasma protein antiserum using crossed immunoelectrophoresis. The twelve peaks that could be recognized in most runs, and that were therefore analyzed in this study, are indicated by stars, and drawn below.

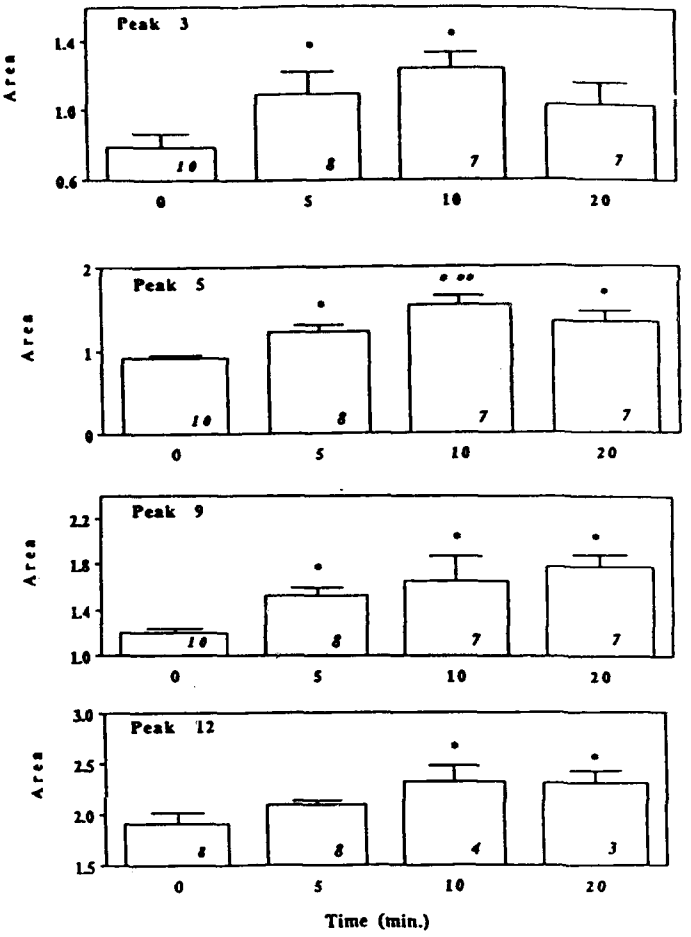

Figure 3. Graphical representation of the peak areas that increased in response to stress. Increases in peak areas on CIEP gels from pooled trout plasma correspond to increases in concentrations of plasma antigens. Mean areas are natural log transformations of the data. Time refers to resting sample pool (0), and rainbow trout kept 5, 10 and 20 min in a shallow bucket after 30 sec in a net in the air. Sample sizes stated in the bars indicate the number of replicate runs in which that peak was measured and included in the analysis of variance. * indicates a significant difference ($p < 0.05$) from the resting sample. ** in Peak 5 indicates that the value for the 10 min pool was significantly higher ($p < 0.05$) than the 5 min value.

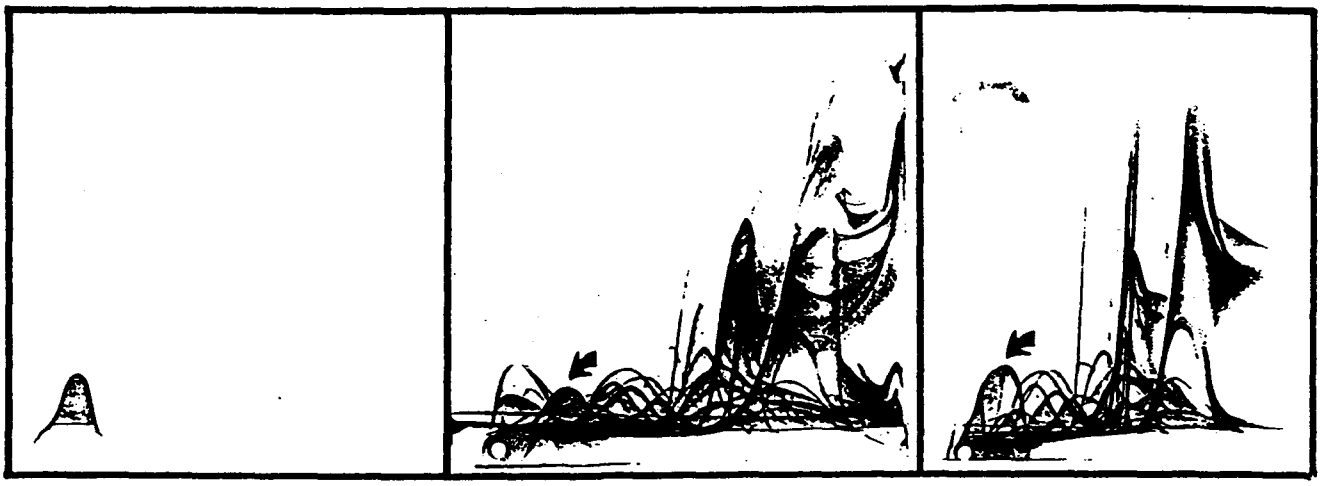

Figure 4. CIEP gels in which Peak 3 (arrow) is identified as C3. **Left:** pure C3 (a gift from Drs. Yano and Kaattari) run under identical CIEP conditions as plasma. **Middle:** plasma from a resting fish, with peak 3 (C3) indicated. **Right:** the same plasma but with the addition of 5 ml (approximately 7 mg) purified trout C3; an increase in size of Peak 3 is evident.

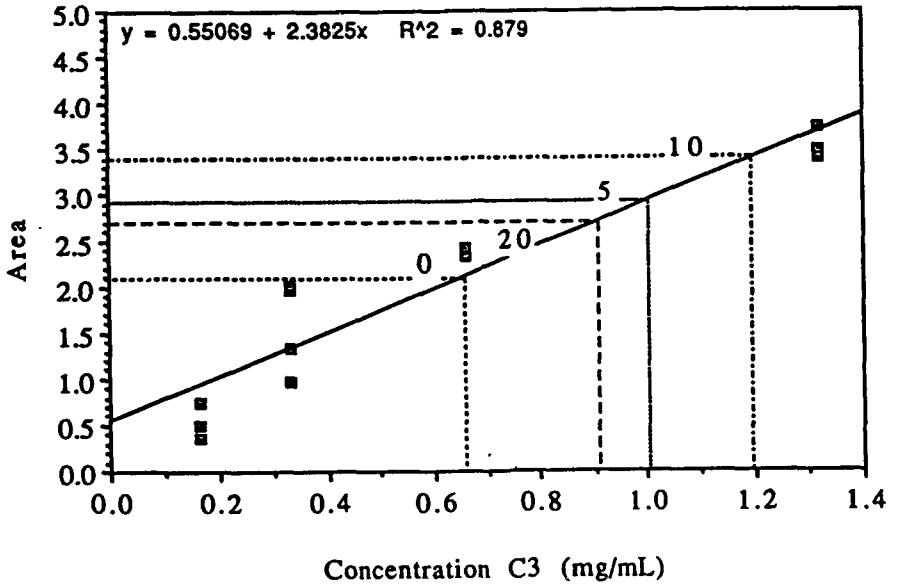

Figure 5. Correlation of C3 concentration and peak areas in CIEP. Horizontal and vertical lines show how C3 concentrations in plasma were derived from mean areas for Peak 3 in trout pooled plasma samples from (0) resting fish, and from fish stressed 5, 10 and 20 min.

DISCUSSION

The speed with which some plasma proteins increase in concentration is remarkable, being faster than could be accounted for by the established mechanisms of the shock response (Koj, 1985). In mice, acute phase sera were found to have significantly increased levels of C3 only after 24 hr (Taktak and Stenning, 1991); in that study no samples were collected until 2 hr after the stress, so any brief increase would not have been detected. The early changes we have observed are almost certainly due to the release of presynthesized molecules. Our observations of increased concentrations of some plasma proteins are consistent not only with intuition, but with the observation (Ruis and Bayne, personal observations) that plasma from acutely stressed rainbow trout has increased opsonic capacity for yeast. The increase in concentration of C3 reported here could be part of the basis for the enhanced opsonic capacity, helping the fish identify invading microorganisms for clearance and destruction by phagocytes, and marking damaged host tissue for removal by the same cells. However, it remains to be determined if other plasma components such as C-reactive protein, fibronectin or mannan-binding protein may contribute to the increase in opsonic capacity of stressed plasma.

CIEP requires large amounts of antiserum, and variations are always observed between gels containing the same samples. Because of these considerations plasma samples were pooled from several fish that had experienced the same stress regime. Each pool was electrophoresed several times in order to obtain values from which (despite run-to-run variance) means could be derived with acceptable precision. The use of pooled sera precluded definitive conclusions about inter-individual differences in concentrations of plasma components. Several CIEP analyses were run on individual serum samples (data not shown), allowing us to conclude that the values obtained with the pooled sera were representative of the group, and not due to single 'outlier' values. Ideal analyses would include several CIEP runs with each of 5 or more **individual** samples, allowing one to determine if all contained similar relative concentrations of plasma components.

The methods used in this study allowed a broad, simultaneous screening for alterations in the concentrations of many individual plasma antigens, without prior knowledge of the identities of the detected molecules. Now that it is clear that several plasma proteins increase in concentration over the time course explored here, it is appropriate to seek identification of those components. Among these, it is likely that one or more are components of the clotting cascade, as the clotting speed of blood is known to increase rapidly in response to trauma (Cassillas and Smith, 1977; Fujikata and Ikeda, 1985; Smit and Schoonbee, 1988).

The identification of C3 in this study illustrates one approach that can be taken to achieve identification of specific plasma proteins. Difficulties we encountered (Demers, 1993) in trying to localize fibronectin and immunoglobulin peaks in our CIEP plates suggest that specific reagents and pure homologous components may be needed for at least some identifications. Others, such as lysozyme and anti-proteases may be identifiable on the basis of activity, if this is retained after immunoprecipitation. It would be natural to focus identification efforts on known components of the clotting cascade, and on the plasma components that are involved in recognition, clearance and/or killing of microbes, including components of the complement system, transferrin, anti-trypsins, α2-macroglobulin, C-reactive protein, mannose-binding protein, fibronectin and others.

It is not clear, in many earlier reports, how closely 'resting' samples of blood approached truly unstressed conditions. Our protocol was designed to approach this as closely as possible, and yielded

cortisol values at the lower end of the reported range. Nearly all studies of plasma cortisol in salmonids have relied on a time-scale more protracted than the one we used (reviewed by Barton and Iwama, 1991). The observation of significant elevation as early as 5.5 min after initiation of stress is, however, consistent with published reports. The delayed kinetics of plasma glucose increase is also consistent with known regulatory effects of cortisol and catecholamines on glycogenolysis and gluconeogenesis.

We do not interpret our results to imply a role for cortisol in the short-term regulation of plasma protein concentrations. Data on plasma concentrations of other stress hormones such as catecholamines and endorphins are needed in order to seek closer correlations with the kinetics of changes we observed. From what is known of the kinetics of catecholamine release (Woodward, 1982), mechanisms of catecholamine action, and the occurrence of adrenergic receptors on hepatocytes (Moon *et al.*, 1988; Ottolenghi *et al.*, 1988; Mommsen *et al.*,1991) and macrophages (Hu *et al.* 1991), catecholamines are more likely candidates for regulating the rapid release of presynthesized plasma proteins to yield the changes we have reported.

The biological relevance of our results needs to be addressed by means of whole-fish bioassays. Fish should be challenged with live microbes at intervals of a few min after stress. Suitable indications of potentiation within the innate immune system would include enhanced rates of bacterial clearance and killing, and the ability of the fish to survive pathogens. It will also be interesting to determine the identities of responding plasma proteins, to discover the source(s) of presynthesized plasma proteins, and to determine their physiologically active releasers. Our efforts are directed at hepatic parenchymal cells as sources, and catecholamines, endorphins and LPS as releasers.

In summary, cortisol and several plasma proteins in rainbow trout experienced increases in concentration within 5.5 min of the initiation of an acute stress. This subpopulation included at least one protein (C3) that is involved in both lytic and opsonic functions. This supports the idea that constitutive internal defenses are potentiated when the earliest stress hormones to increase in the plasma (catecholamines) prepare an animal for 'fight or flight'.

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Chapter 2

The Effects of Methyl Parathion on Phagocytosis and Respiratory Burst Activity of Tiger Shrimp (*Penaeus monodon*) Phagocytes

Nantarika Bodhipaksha[1] and Beverly Anne Weeks-Perkins[2]

[1]Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
and
[2]School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Va. 23062, USA
[2] for Correspondence

ABSTRACT

Phagocytic activity (percent phagocytosis), the phagocytic index, and superoxide anion production by circulating and cardiac phagocytes collected from Giant Black Tiger shrimp, (*Penaeus monodon*), were measured after a 96 hr exposure to varying concentrations (0, 1, 2, 3, 6 and 10 ppb) of the organophosphorous pesticide, methyl parathion. For both circulating and cardiac phagocytes, there was a decreasing trend in the phagocytosis of yeast cells as the concentration of methyl parathion was increased. Exposure to methyl parathion at and above 2 ppb resulted in a significant reduction in phagocytic activity for both cell types as compared to the control group. The phagocytic index for circulating and cardiac cells showed a decreasing trend with increasing concentrations of pesticide with significant differences at and above 6 ppb. In measuring the reduction of nitroblue tetrazolium (NBT) as an indicator of superoxide production, it was found that there were no significant differences between circulating phagocytes in the control group and the groups exposed to 1 and 2 ppb methyl parathion. Both circulating and cardiac phagocytes exhibited significant increases in superoxide production at 6 and 10 ppb, as well as at 3 ppb in the case of cardiac phagocytes. For all concentrations tested, superoxide production was significantly greater for circulating phagocytes than for cardiac phagocytes.

INTRODUCTION

Phagocytosis is the primary defense mechanism against invasion of pathogenic organisms in invertebrates (Olivier *et al.*, 1988; Robohm, 1984). However, little is known about the intracellular mechanisms involved in the destruction of phagocytized microorganisms in decapods such as shrimp. Phagocytic hemocytes are believed to be the predominant cellular immune defense agents in crustaceans, where phagocytosis has been observed *in vivo* in circulating phagocytes and

in phagocytes located within the heart muscle and liver tissue (Paterson and Stewart, 1974). The small granular hemocytes, some of which contain lysosomal enzymes, are the primary cells involved in the phagocytosis of foreign particles in shrimp. Various enzymes such as acid phosphatase, esterase and beta-glucuronidase have been found in the cytoplasmic vesicles within these cells. Large granular hemocytes are also capable of phagocytizing foreign material but do so with less frequency than the small granular hemocytes (Hose and Martin, 1989).

In decapods, some cardiac cells also have phagocytic capabilities (Johnson, 1987). Their ability to clear toxic substances through pinocytosis has been demonstrated (Ghiretti-Magaldi *et al.*, 1977), but little is known about the effects of pesticide exposure on the phagocytic ability of circulating and cardiac phagocytes.

Methyl parathion is an organophosphorous pesticide that functions by inhibiting acetylcholinesterase and pseudocholinesterase in insects. Methyl parathion is metabolized into methyl paraoxon which reacts with acetylcholinesterase at esteric sites and causes a phosphorylated enzyme complex to form. This complex acts as an irreversible cholinesterase inhibitor. As a result, the hydrolysis of acetylcholine is inhibited and organic processes, including immunological responses, may be impaired.

Phase one of this study was to determine if methyl parathion is capable of adversely affecting the phagocytic ability (*e.g.* percent phagocytosis and phagocytic index) of shrimp phagocytes. The second phase was to measure the effect of methyl parathion on the production of superoxide anions (O_2^-). Because stimulation of the hemocyte cell membrane during phagocytosis triggers the production of microbicidal oxygen free radicals or reactive oxygen species (O_2^-, H_2O_2, OH^-, 'O), the quantification of the O_2^- produced by phagocytes can serve as a measure of the respiratory burst (Secombes, 1990).

The respiratory burst has been studied extensively in fish macrophages (Chung and Secombes, 1988; Secombes *et al.*, 1988; Warinner *et al.*, 1988; Anderson *et al.*, 1990; Mathews *et al.*, 1990; Rice and Weeks-Perkins, 1991; Zelikoff *et al.*, 1991; Zelikoff and Enane, 1991; Kelly-Reay and Weeks-Perkins, in press). The stimulation produced by the phagocytic process in fish macrophages involves the hexose monophosphate shunt (HMP) and nicotinamide adenine dinucleotide-2'-phosphate (NADPH) oxidase which triggers the production of reactive oxygen species. In the membrane, intracellular superoxide anion radicals (O_2^-), one component of the respiratory burst, are produced (Secombes, 1990).

Stimulation of phagocytes at the membrane level can be achieved by using a soluble activating agent such as phorbol myristate acetate (PMA). Hydrogen peroxide, hydroxyl ions and singlet oxygen are produced from the superoxide which is generated upon stimulation. The production of hydrogen peroxide is catalyzed by the enzyme, superoxide dismutase (SOD) (Findovich, 1978). The production of these antimicrobial agents within phagocytic cells prevents invading foreign agents from damaging body systems.

Intracellular superoxide (O_2^-) can be measured *in vitro* by quantifying the reduction of the redox dye, nitroblue tetrazolium (NBT) into a water insoluble blue formazan which can be read with a spectrophotometer after dissolving in potassium hydroxide (KOH) and dimethyl sulfoxide (DMSO). Superoxide production can be verified by preventing its reduction with exogenous SOD which dismutates O_2^- and generates H_2O_2 (Secombes *et al.*, 1988). The optimum PMA concentra-

tion and incubation time are unique for each species and must therefore be determined individually (Secombes, 1990). The optimum range of PMA for the production of intracellular O_2^- was 1 µg/mL for shrimp phagocytes which corresponds to the optimum range of 0.2-2 µg/mL for superoxide production in fish macrophages.

Methyl parathion is an organophosphorous pesticide whose widespread use increases the opportunity for water supply contamination. Thus far, studies conducted on the effects of this pesticide on aquatic fauna have focused on enzyme systems, concentrating on the inhibition of acetylcholinesterase (Corbett, 1974). However, since fish macrophages have been shown to have reduced phagocytosis and reactive oxygen species production when exposed to pollutants such as polynuclear aromatic hydrocarbons (PAH) (Weeks and Warinner, 1984; Warinner *et al.*, 1988; Weeks-Perkins and Wong-Verelle, in preparation), this study focuses on the effects of methyl parathion on phagocytosis and respiratory burst activity in tiger shrimp.

MATERIALS AND METHODS

Animals

Giant Black Tiger shrimp (*Penaeus monodon*), approximately four months old, were collected from a commercial earth pond in Samutsakorn, Thailand. The average weight and length of the shrimp were 35 grams and 13 cm, respectively. The shrimp were maintained in an aerated static system consisting of 30 L aquaria held at 20 ppt salinity, 25°C and pH 8.0. Exposure to methyl parathion was performed by adding methyl parathion in methanol (1, 2, 3, 6 and 10 ppb) to the holding tanks. In the control group, 3 mL of methanol alone was added. At 96 hr, surviving shrimp were sacrificed by placing them on ice.

Survival rate determination

After exposure to the pesticide for 96 hr at different concentrations of methyl parathion (0, 1, 2, 3, 6 and 10 ppb), surviving shrimp were counted and the LC_{50} was calculated. Four replicates were performed.

Phagocyte Isolation

Circulating phagocytes

Hemolymph was drawn from the ventral sinus of shrimp using a 5 mL syringe with a 20 gauge needle. Cooled hemolymph anticoagulation medium (HAM) supplemented with 2% fetal calf serum (FCS) and L-15 medium (Bodhipaksha, 1994) was first placed in the syringe at a ratio of 1 mL/1.5 mL hemolymph before drawing the hemolymph from the sinus cavity. The syringe containing hemocytes and HAM was gently mixed to prevent clotting. Viability of the cells was determined by trypan blue exclusion, and cells were adjusted to a concentration of $1x10^6$ cells/mL using HAM.

Cardiac phagocytes

Hearts were aseptically removed and transferred to HAM supplemented with 500 U/mL penicillin, 0.3 mg/mL streptomycin and 10% FCS (Sharp *et al.*, 1991). Cell suspensions were prepared by teasing the tissue with a glass rod over a stainless steel mesh screen in a petri dish. Cell suspensions were collected, centrifuged at 400xg for 5 min., and resuspended in supplemented HAM at a concentration of 1 x 10^6 cells/mL. Phagocytes were used in the phagocytosis and NBT assays immediately after collection.

Opsonization of yeast cells

Commercial Baker's yeast (*Saccharomyces cerevisiae*) was prepared for phagocytosis assays by suspending 1.5 g in 7 mL phosphate buffered saline (PBS) and autoclaving the suspension at 120°C and 15 psi for 15 min. The yeast cells were rendered non-viable so that a uniform preparation of yeast cells were available in all experiments (Bodhipaksha *et al.*, 1992). After two washes in PBS, the yeast cells were resuspended in HAM at a 1% packed suspension (Fryer and Bayne, 1990). Just prior to use, cells were resuspended at 8.0 x 10^7/mL in HAM.

Opsonization of antigen in plasma has been shown to increase phagocytosis (Goldenberg and Greenberg, 1983). Yeast cells were incubated for 1 hr in shrimp serum which was obtained from the hemolymph drawn from the ventral sinus and centrifuged at 1500 rpm for 5 min to separate the hemocytes from the serum. The yeast cells were then washed twice with PBS and centrifuged at 2000 rpm for 5 min before being resuspended in HAM at a concentration of 1% packed suspension. The opsonized killed yeast cell suspension was freshly made before each experiment to avoid any alteration in the opsonic property during storage (Fryer and Bayne, 1989).

Prior to conducting phagocytic assays, yeast cells were stained with the fluorescent dye, phloxine B(Sigma) solution (10% in PBS). This was used to facilitate microscopic examination of phagocytized yeast cells using light and fluorescent microscopy.

Phagocytosis assays

The phagocyte suspensions were placed onto glass slides (100 µL/slide), and the cells were allowed to adhere for 30 min. The slides with attached phagocyte monolayers were rinsed gently with HAM to remove unattached phagocytes. Opsonized yeast cell suspensions (50 µL) were overlaid on each phagocyte monolayer (providing a yeast cell to phagocyte ratio of 50 to 1). After gently mixing with a pipette, the slides were incubated at room temperature in a humidity chamber for 90 min. Non-phagocytized yeast cells were removed by washing each slide twice with 100 µl of HAM using a pipette.

To facilitate quantification of phagocytized yeast cells, 100 µL of trypsin solution (0.2-0.5% trypsin dissolved in HAM and adjusted to pH 7.6) was dropped on each slide for 1 min to loosen adsorbed, but non-phagocytized yeast cells, before rinsing in HAM. This step in the process improved accuracy in counting since only phagocytized yeast cells remained on the slides.

Slide preparations were fixed with Davidson's fixative (Shaw and Battle, 1957) for 10 min, rinsed with HAM, air dried and stained with Hematology Three Step Stain (Accra, N.J.) and phloxine. The phagocytized yeast cells were easily observed using fluorescent microscopy and distinguished

from other organelles or foreign particles using morphological criteria. Phagocytized yeast cells appeared to be at a focal level similar to the phagocytes. Two hundred phagocytes were counted and characterized as being active (containing phagocytized yeast cells) or non-active (not containing yeast cells). The individual yeast cells within active phagocytes were also counted. The percent phagocytosis (number of active phagocytes/100 phagocytes) and the phagocytic index (number of yeast cells phagocytized/phagocyte) were determined for both circulating and cardiac phagocytes. Four replicates were performed.

Superoxide anion detection

The detection of intracellular superoxide anion (O_2^-) which has been accepted as a direct and accurate method of quantifying the intensity of the respiratory burst was detected by reduction of the redox dye, nitroblue tetrazolium (NBT) (Secombes, 1990).

Phagocyte monolayers were prepared in microtitre plates (NUNC) by adding 100 µL of hemolymph or cardiac cell suspension to each well. The plates were incubated for 2 hr at room temperature and unattached cells were then removed by washing with L-15 medium.

One hundred µL of a solution containing 1 mg/mL NBT dissolved in L-15 medium and 1 µg/mL PMA were added to each of the wells. The plates were incubated for 30 min at 25°C. Superoxide dismutase (SOD) was added to some of the NBT-PMA solution and placed in the wells as a control. During this incubation, NBT was reduced by O_2^- into a water insoluble blue formazan. After removal of the excess medium, 70% methanol was used to fix the cells for 10 min. The wells were then washed with methanol 3 times and allowed to air-dry. To dissolve the formazan, 120 µL of 2 M KOH and 140 µL of dimethyl sulfoxide (DMSO) were added to each well and mixed with a pipette. The concentration of the turquoise blue colored solution was measured in a multiscan spectrophotometer (Dynatech MR5000) at 630 nm using KOH/DMSO as a blank.

Statistical analysis

The statistical analysis of data was performed using analysis of variance for determination of significant differences in values for control and exposed groups of fish. The least signifant difference (LSD) method of multiple comparisons was used to identify significantly different responses among treatment groups (Dowdy and Wearden, 1991).

RESULTS

Survival rate

The survival rate of shrimp exposed to methyl parathion was decreased with increasing concentrations of pesticide. LC_{50} was calculated to be approximately 3 ppb. Surviving shrimp showed decoloration of the body and were very weak, especially those exposed to concentrations higher than 2 ppb.

Morphological studies

Morphology of phagocytes in phagocytosis assays was observed using light microscopy. The phagocytes formed numerous filopodia and showed exocytosis of granules. The circulating hemocytes, when they were freshly removed from the animal, were observed to have a round shape. However, when attached to the surface of glass slides, the cells spread out, became flattened, and extended pseudopodia. Sminia and Van der Knaap (1986) suggested that cells with a high nuclear to cytoplasmic ratio and many ribosomes may not spread. In this study, the spreading cells usually had a higher cytoplasmic to nuclear ratio than non-spreading ones. The round hemocytes exhibited less phagocytic ability than the spreading ones. Spreading hemocytes which had attached to the glass slides were studied in this experiment. Phagocytosis of yeast cells occurred following opsonization by incubating the cells with hemolymph (Soderhall and Smith, 1986). Opsonins in shrimp exists in the form of proenzymes in the small granular and large granular phagocytic cells. Phagocytosis was initiated when the yeast cells came in contact with hemocytes. Autoclaving the cells at 120°C, 15 psi for 15 min killed the yeast cells but their non-self recognition was maintained (Bodhipaksha *et al.*, 1992).

Phagocytosis Assays

Results of phagocytosis (percent phagocytosis) assays for circulating and cardiac phagocytes are shown in Figure 1. Percent phagocytosis for cardiac phagocytes decreased significantly ($p \leq 0.05$) in all treatment groups (1, 2, 3, 6 and 10 ppb) as compared to the control. For circulating phagocytes there was a *significant ($p \leq 0.05$) decrease in phagocytosis at 2, 3, 6, and 10 ppb as compared to the* control group. In each treatment group, there were no significant differences between circulating and cardiac phagocytic activity.

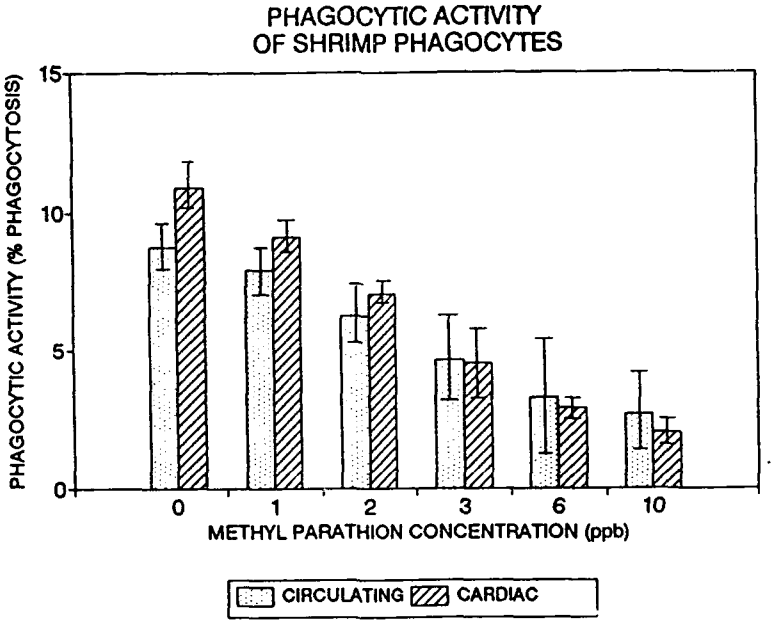

Figure 1. Determination of percent phagocytosis as a measure of phagocytic ability of circulating and cardiac phagocytes in shrimp exposed to methyl parathion. The results of the means of four experiments are shown with error bars representing the standard deviations of the mean.

The results of the phagocytic index assays for both circulating and cardiac phagocytes (Figure 2) indicated the presence of a decreasing trend as the methyl parathion concentration was increased from 0 to 10 ppb. The decreases were significant ($p \leq 0.05$) for 3, 6 and 10 ppb as compared to the control group.

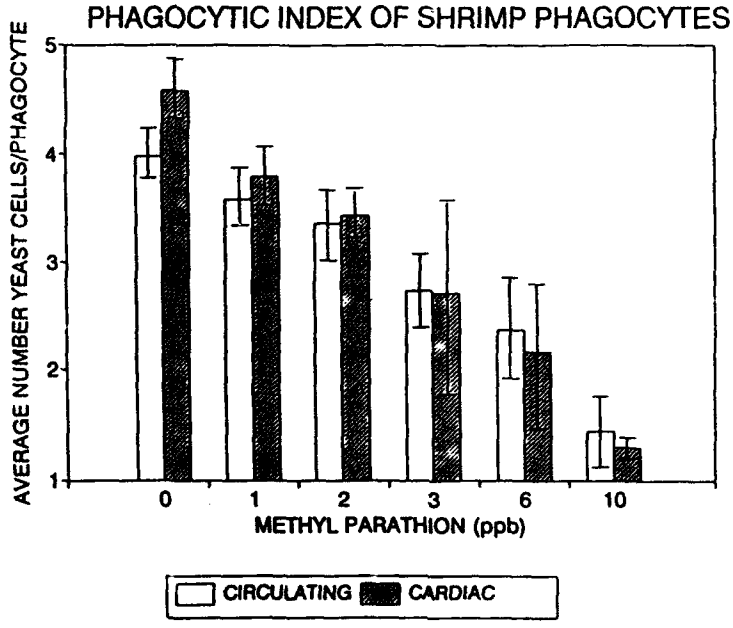

Figure 2. The phagocytic index (average number of yeast cells/phagocyte) of circulating and cardiac phagocytes in shrimp exposed to methyl parathion. The results of the means of four experiments are shown with error bars representing the standard deviations of the mean.

Respiratory burst assays

Superoxide anions were released during the respiratory burst by circulating and cardiac phagocytes after the shrimp were exposed to methyl parathion for 96 hr (Figure 3). For circulating phagocytes, no significant differences were found between the control group and the groups exposed to 1, 2 and 3 ppb methyl parathion. For cardiac phagocytes, the experimental group exposed to 3 ppb, but not to 1 and 2 ppb, showed a significant ($p \leq 0.05$) increase in superoxide production as compared to the control group. In shrimp exposed to 6 and 10 ppb, significant ($p \leq 0.05$) increases in superoxide anion production were found in both circulating and cardiac phagocytes as compared to the control. Cardiac and circulating phagocytes showed similar increasing trends in respiratory burst activity when shrimp were exposed to methyl parathion. However, for all groups, superoxide production was significantly ($p \leq 0.05$) greater for circulating than for cardiac phagocytes.

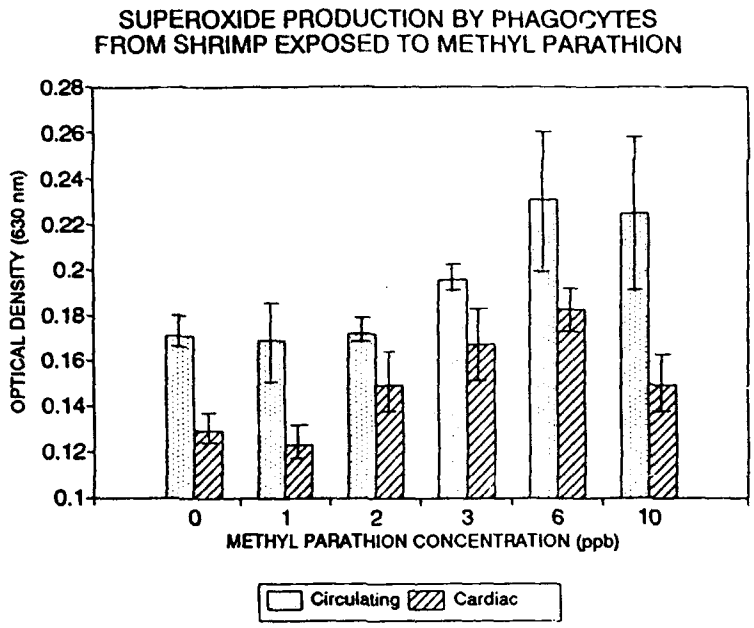

Figure 3. The reduction of nitroblue tetrazolium (NBT) to determine superoxide production by circulating and cardiac phagocytes from methyl parathion-exposed shrimp. The results of the means of four experiments are shown with error bars representing the standard deviations of the mean.

DISCUSSION

The phagocytic ability of phagocytes from shrimp exposed to methyl parathion was studied using immunological assays for phagocytosis. Phagocytosis is an important part of the cellular immune system and functions to protect the host by destroying infectious agents. The results of this study demonstrated that methyl parathion exposure caused a siginificant decrease in the percent phagocytosis for circulating and cardiac shrimp phagocytes. In these experiments, the average percent phagocytosis in the control group was 8.75% for circulating hemocytes and 10.88% for cardiac phagocytes.

Opsonization of yeast cells with cell-free hemolymph as demonstrated by Hose and Martin (1989) was shown to significantly increase maximal phagocytic rates in small granular cells. Opsonic factors in crustaceans are thought to be either products of an opsonizing activating system (Smith and Soderhall, 1983; Soderhall *et al.*, 1986) or agglutinins, specifically lectins. Some of the carbohydrate specificities of crustacean opsonins were defined by Goldenberg and Greenberg (1983), who showed that D(+) glucose and D(+) mannose caused a significant inhibition of the opsonins and decreased the percent phagocytosis by hemocytes. Methyl parathion exposure at sublethal concentrations may cause changes in glucose metabolism of invertebrate cells (Moorthy *et al.*, 1985).

After freshwater mussels were exposed to methyl parathion, glucose-6-phosphate dehydrogenase activity increased, suggesting enhanced oxidation of glucose and reduction in the aerobic oxidation of glucose (Moorthy *et al.*, 1985). In the same manner, this exposure may cause an increase in cell metabolites (Hoar, 1983) and a decrease in non-self recognition necessary for phagocytosis. In this study, the percent phagocytosis by hemocytes decreased with an increase in exposure to methyl parathion. Also, since methyl parathion has been shown to cause both abnormalities in mitochondrial oxidative metabolism and a decrease in ATPases (Moorthy *et al.*, 1985), the energy produced

for phagocytic activity was probably decreased; and therefore, there was a decline in the number of cells capable of phagocytosis. Another possible factor that could lower the amount of energy that hemocytes can use for phagocytosis is an elevation in serum glucose induced by the stress of pesticide exposure for 96 hr. Lynch (1973) indicated that elevation of serum glucose is an indicator of stress in the crab, *Callinectes sapidus*.

The attempt to quantify phagocytosis accurately depends on the removal of all loose and adsorbed particles (Prowse and Tait, 1969). Adsorption of opsonized or untreated particles to hemocytes is sufficiently strong so that washing alone does not remove adsorbed particles. In describing the effect of trypsin on the adsorption of particles by invertebrate hemocytes, Scott (1971) showed that adsorption depends on a protein receptor site. Therefore trypsin was used with success, in these experiments to eliminate shrimp hemocyte adsorption.

The phagocytic index (average no. of yeast cells/phagocyte) also showed significant decreases with increasing concentrations of methyl parathion for both circulating and cardiac phagocytes. Further work will be required to determine the mechanism of impaired phagocytosis caused by methyl parathion.

This study confirmed that circulating and cardiac phagocytes collected from shrimp responded to the membrane activating agent PMA and produced superoxide anions (O_2^-) during the respiratory burst. After exposure to methyl parathion, shrimp exposed to 6, and 10 ppb showed significant increases in superoxide anion production for circulating and 3, 6 and 10 ppb cardiac phagocytes. In freshwater mussels exposed to methyl parathion, Moorthy *et al.* (1985) found an increase in glucose-6-phosphate dehydrogenase activity suggesting enhanced oxidation of glucose via the hexose monophosphate (HMP) shunt. The HMP shunt generates NADPH which provides the one electron necessary for the reduction of oxygen into superoxide. Therefore, the increases in superoxide may be attributed to an elevation in glucose-6-phosphate dehydrogenase activity. This suggests that phagocytes in shrimp exposed to methyl parathion may have undergone increased glucose oxidation via the HMP shunt, and the decreased aerobic oxidation of glucose induced higher O_2^- production by phagocytes following stimulation with PMA.

Enzymatic activities, especially microsomal enzymes (Corbett, 1974) are particularly important in determining toxicity tolerance to organophosphorous pesticides in invertebrates. These enzymes can metabolize pesticides into non-toxic substances, especially those which are lipid soluble. Further investigation as to how cellular enzymes affect phagocytosis and superoxide production is needed.

Phagocytosis and respiratory burst assays have the potential to be used in shrimp as biomarkers to assess organophosphorous pesticide contamination in the environment.

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Chapter 3

Cytometric Parameters of Bivalve Molluscs: Effect of Environmental Factors

Michel Auffret and Radouane Oubella

Institute for Marine Studies, URA CNRS D1513
University of West Brittany, BP 452
29279 BREST Cedex, France

ABSTRACT

Among aquatic organisms, molluscs are being used more and more in monitoring programs of marine ecosystems. They constitute abundant populations on the coasts of every area in the world and often inhabit estuaries where variations of environmental factors, including anthropic contamination are the most pronounced. As filter feeding organisms, they may enhance pollution effects by bioaccumulating various xenobiotics. The immune defense system of bivalve molluscs relies, as in other invertebrates, on cellular and humoral factors; the former considered most important in the defense against microorganisms, The various steps in the cellular immune response are susceptible to alteration by environmental factors. These are potential tools for monitoring studies.

The cytometric parameters in hemolymph are total circulating cell and differential counts. We have shown that the former is correlated to individual mass of soft tissues. This relation was found to vary with the origin of the population of the individual cells. In addition, absolute counts within populations varied according to seasonal cycles which may in turn be related to biological cycles.

Experimental work was designed to : 1) Identify cellular responses of the immunodefense system in two bivalve species (*Crassostrea gigas* and *Ruditapes philippinarum*) and 2) identify biotic or abiotic stimuli able to modify these responses.The only factor that lowered hemocyte density in *Ruditapes philippinarum* was starvation. This species challenged with a pathogenic bacteria (*Vibrio* P1) showed increased hemocyte density after 72 hr. This response was not seen with a non-pathogenic bacterium or with killed pathogenic bacterium. *Crassostrea gigas* exposed to high doses of metal in the laboratory showed increased hemocyte density after one week. Differential counts showed that changes occurred in the hemocyte subpopulations.

The observed changes in cytometric parameters have been interpreted either as immune responses or as consequences of hemolymph cell alteration. Quantification of such parameters could be introduced in monitoring studies as sensitive biomarkers for the status of the immune defense system in molluscs.

INTRODUCTION

Bivalve molluscs have biological and ecological characteristics that expose them to various stresses originating from often acute changes of environmental factors, including salinity, temperature, as well as microbial challenge. Chemical contamination is of particular importance since bivalves, as filter-feeding organisms, bioaccumulate various micro-contaminants from their environment. Abundant populations of bivalve molluscs often inhabit coastal ecosystems including estuaries and some of these species are used as sentinel organisms in environmental monitoring studies. As in other aquatic organisms, the immune system of molluscs is a target of many external stressors that may cause immunosuppression. Since the pioneering work of Feng (1965) on temperature effects, it has been shown that hemolymph cytometric parameters vary in bivalve molluscs following stressor exposure. It was reported (Cheng and Sullivan, 1984, Cheng, 1988) that metal ion exposure could induce variations in hemocyte subpopulation composition in a way depending on metal ions. According to Suresh and Mohandas (1987), metal ion exposure depressed total hemocyte counts but this response was modulated by the environmental conditions of the molluscs. Organic contamination effects were observed to depend on the contaminant. Although Anderson *et al.* (1981) found that total and differential hemocyte counts in clams exposed to chlorinated hydrocarbons were unaffected, contamination by hydrocarbons modified cytometric parameters in mussels and oysters (Mc Cormick-Ray, 1987; Sami *et al.*. 1992). According to Pickwell and Steinert (1988), and Fisher *et al.* (1990) exposure of mussels and oysters to the organotin toxicant, tributyltin, induced cellular responses, as seen with metal contamination. Among biotic environmental factors, bacterial contamination is very frequent, even in the absence of true disease. Experimental challenge of clams with pathogenic bacteria has been found to increase total hemocyte counts (Oubella *et al.*, 1993).

Many recent studies attest to the fact that the immune defenses of bivalve molluscs are becoming a major field of research with multiple applications to environmental monitoring. This paper presents observations and results of experimental work to design biological tools for studying immune responses of two species of bivalve molluscs, the littleneck clam (*Ruditapes philippinarum*) and the Pacific oyster (*Crassostrea gigas*). Both species are found on European Atlantic coasts as cultured and wild populations which inhabit estuaries and other ecosystems exposed to anthropic pollution.

REVIEW OF RESULTS

Cytometric parameters of bivalve molluscan hemolymph cells

Bivalve molluscs have open circulatory systems consisting of a heart, vessels of various caliber and a complex network of hemolymph sinuses localized in organs and adductor muscles. A major consequence is that only a fraction of the hemolymph flows in the vessels at a given time, defining a circulatory compartment. Hemolymph samples are most often withdrawn from the adductor muscle sinus through a notch or a drill in the shell edge.

On a morphological and cytological basis, most of bivalve molluscs possess two major types of hemocytes; granulocytes and agranular hemocytes (Auffret, 1988). The latter have also been termed hyalinocytes or macrophages. All the species possess these cells. Granulocytes appear as different cell types which are characterized by their cytoplasmic granules. The oyster *Crassostrea gigas*

possesses basophilic and acidophilic granulocytes. The clam *Ruditapes philippinarum* has only neutrophilic granulocytes. Other minor cell types may be observed in hemolymph, particularly small, undifferentiated cells and vacuolized cells. The immune function of bivalve hemocytes has been reviewed by Fisher (1986) and Feng (1988). These cells play a major role based on their nonspecific phagocytic activity against microorganisms and various pathogens..

Circulating cell subpopulations are most often identified upon cytological criteria. In the species studied here, large agranular hemocytes represent more than the half of circulating cells (Figure 1).

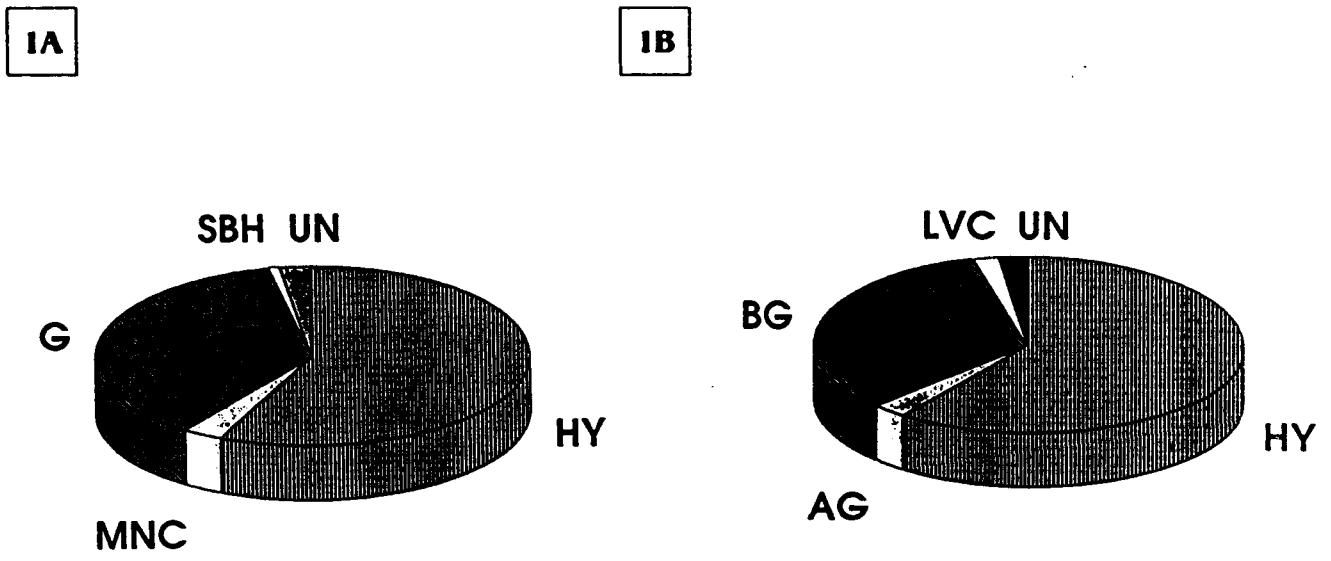

Figure 1. Circulating cell subpopulation rates after differential counts in the clam *Ruditapes philippinarum* (a) and the oyster *Crassostrea gigas* (b). Cell monolayers were made by cytocentrifugation and May-Grunwald-Giemsa staining. Values are means from several individuals from healthy populations. **HY** = hyalinocytes; **G** = granulocytes; **BG** = basophilic granulocytes; **AG** = acidophilic granulocytes; **SBH** = small basophilic hemocytes; **LVC** = large vacuolated cells; **MNC** = multinucleated cells; **UN** = unidentified cells

Flow cytometric analysis of circulating bivalve hemocytes reveal no relationship between size, structure and cell type (Figure 2). This situation, in addition to the occurence of a great number of small cells, supports the idea that cell maturation occurs in the circulating compartment. No sites for hemocytopoiesis have been identified in these animals and an accepted hypothesis is that blood cells originate from tissues such as interstitial, mesenchymatous tissues.

In bivalve molluscs, great variability has been observed in the numbers of total circulating cells between individuals from different populations, as well as within populations. It was demonstrated that this parameter may be greatly influenced by physico-chemical (*i.e.* temperature) and physiological factors (*i.e.* heart rate) (Feng, 1965). Recent observations in several populations of *C. gigas* and *R. philippinarum* have shown that hemocyte density may be correlated to the dry weight of

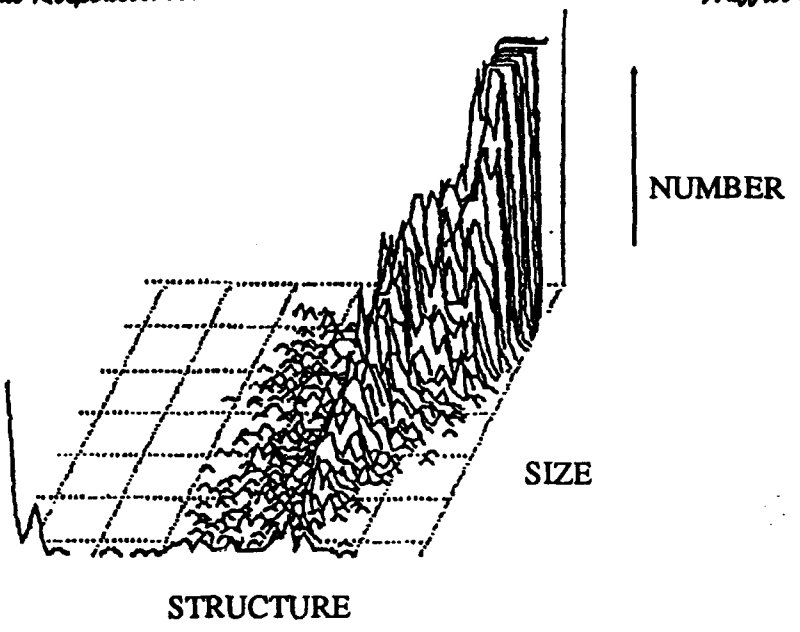

Figure 2 Flow cytometry analysis of circulating cells from the oyster *Crassostrea gigas*. Structure, size and number are plotted in this three-dimensional diagram displaying a conspicuously, high number of small cells.

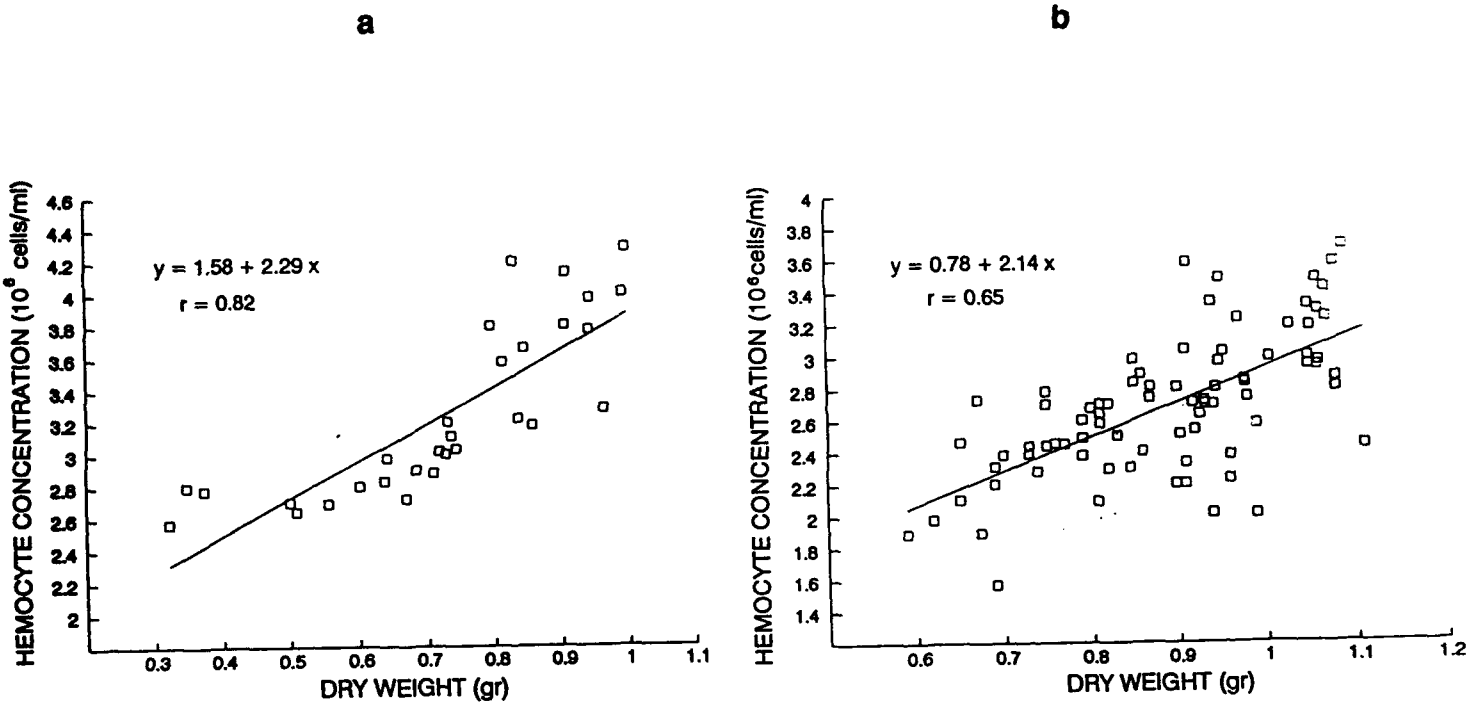

Figure 3 a ,b. Scatter plot of hemocyte concentration versus soft tissue dry weight in healthy, reared populations of *R. philippinarum* . These diagrams show a positive, highly significant correlation between these two parameters .

a. Cultured population from the Channel coast (Landeda). n = 30, r = 0.82, p <.01 for *t* analysis of regression weight.

b. Cumulated values from four cultured populations from the Atlantic coast (Arachon Bay). n = 80, r = 0.65, p < 0.01 for *t* analysis of regression weight

soft tissues. Thus using a ratio of hemocyte concentration/dry weight may reduce the variability observed (Figure 3).

The calculated coefficient of determination however varied among populations. The occurrence of low levels of correlation has not been explained yet but it could reveal heterogeneity within populations. No correlation with a condition index (e.g. ratio of soft tissue weight to shell size) appeared in most of the populations analyzed. Stumpf and Gilbertson (1978) found a comparable correlation between shell size and hemocyte concentration in a gastropod mollusc. An explanation of this uncommon characteristic could be a combination of biological features such as continuous production of circulating cells, long cell life-span and rapid growth of tissues. Both changes in hemocyte numbers and internal variability of hemocyte concentration among individuals may have physiological significance. Increased or decreased circulating cell numbers reflect responses of the immune system. Otherwise, increased variability in observed hemocyte counts may reveal physiological diversity in the ability to produce the cellular response but also, basic, possibly genetical, responsive capacity affecting the susceptibility of individuals to pathogens. By explaining in part the observed variability in hemocyte concentration, the relationship shown above opens an interesting field in analyzing deviations of this cytometric parameter from expected values. Furthermore, in addition to the limitations emphasized above, it underlines the risk of using single-individual analysis. Total hemocyte counts should rather be performed on a population basis in samples exhibiting homogenous physiological conditions.

Variations in cytometric parameters

Variations in numbers of circulating cells involved in non-self defense are among the early signs of triggering cellular immune systems. In bivalve molluscs, the major role played by hemocytes in internal defense has suggested that circulating cell concentration and subpopulation percentages could also be effective parameters to probe the functioning of their immunodefense system. A series of observations and experiments were designed to identify responses and responsible factors in hemocyte macrophage function with respect to other biological functions.

Seasonal changes

A one-year survey of three wild populations of *C. gigas* showed that hemocyte counts/ml varied on a seasonal basis (Figure 4).

Comparable profiles were observed in all three populations. The observed cyclic changes may result from dramatic internal changes linked to other biological functions. For example reproduction in these organisms requires great metabolite mobilization via the hemolymph. Nutritional processes which also require metabolic activity, may involve cellular and humoral hemolymph components. These metabolic requirements vary since food availability for filter-feeding organisms follows an acute seasonal cycle.

Experimental acute salinity changes

After an acclimation period, clams, *R. philippinarum*, were exposed abruptly to either high or low salinity (32% to 15% and 15% to 32%) for 7 days. The number of circulating hemocytes was not altered even if humoral parameters commonly involved in osmolyte regulation (free amino acids, peptidases) varied. The primary effects of salinity effects on bivalve hemocytes were related to cell

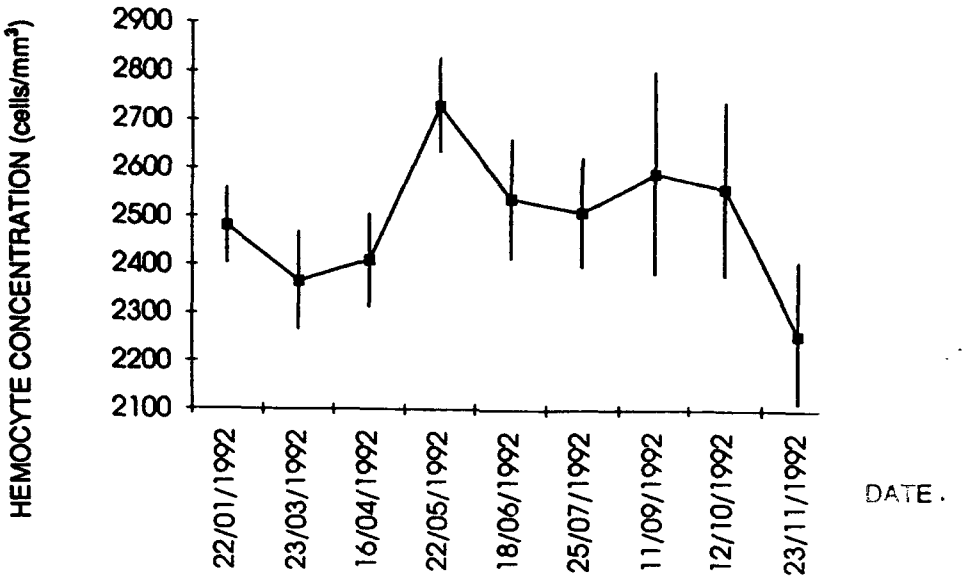

Figure 4. Seasonal variation of the mean hemocyte concentration in a wild population of *C. gigas* from the Atlantic coast (Rade de Brest). Vertical bars represent the standard error of the mean.

activity, as shown by *in vitro* experiments. These affected time for spreading, locomotion rate and particle-binding rate (Fisher and Newell, 1986; Fisher *et al.*, 1987).

Starvation

One week of fasting resulted in a 65% decrease in hemocyte density in *R. philippinarum* (Table 1) (Oubella *et al.*, 1993).

Table 1
Variation of mean hemocyte concentration (10^6cells/ml) in *R. Philippinarum* starved for 1 week compared to individuals fed daily with cultured algae.

T_0	Starved	Fed
2.80 (0.06)	1.75 (0.06) (*)	2.72 (0.07)

Numbers in parenthesis are standard error (n=20) (*): significant difference ($p<0.01$) compared to 'fed' individuals (Student t test)

Further experiments showed that the hemocyte density increased after feeding resumed. This rapid increase supports the idea that changes in number of circulating cells result from reversible mobilization of hemocytes between tissues and the circulatory system (Mounkassa and Jourdane, 1990; Oubella *et al.*, 1993). A comparable response to starvation stress has been observed in other invertebrates, such as as crustaceans (Stewart *et al.*, 1967; Bauchau and Plaquet, 1973). Involved

stimuli could be the metabolic processes that change the make-up of hemolymph plasma. Nevertheless, the magnitude of the decreases in circulating cell number indicate tremendous physiological changes in these organisms, and these changes may interfere with the efficiency of cellular immune responses.

Experimental pathogenic challenge

When *R. philippinarum* were challenged with a pathogenic bacterium (*Vibrio P1*), the number of circulating hemocytes increased by 46% of the initial values after 72 hr (Oubella *et al.*, 1993) (Figure 5).

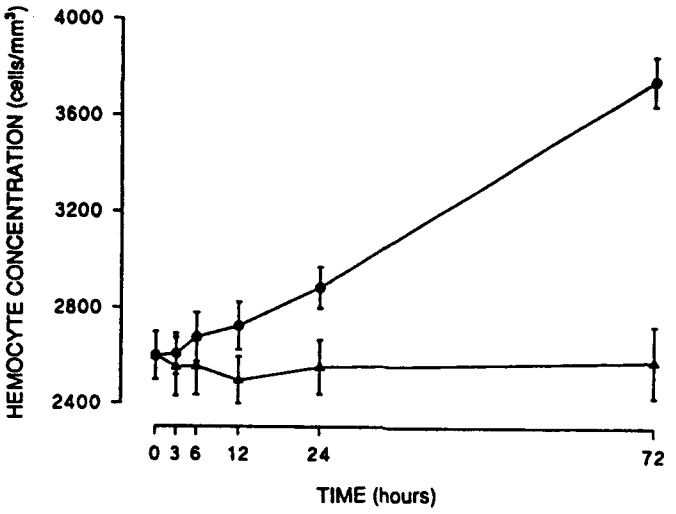

Figure 5. Increase ($p < 0.01$) in hemocyte concentration of *R. philippinarum* after challenge with the pathogenic bacterium *Vibrio P1*. (●). A suspension of bacteria (0.5×10^7) was injected into the intrapallial cavity. Control animals (▲) were injected with filtered sea water. Vertical bars represent the standard error of the mean (n = 20) (Oubella *et al.*, 1993).

An intra-pallial injection containing a minimum of 5×10^6 bacteria was necessary to induce hemocytosis. Heat-killed *Vibrio P1* or non-pathogenic *Vibrio pelagius* did not provoke this increase. The hemocytosis observed in the clams appeared to be a response of their immune system. These results improved the understanding of dynamics and stimulating factors of antibacterial defense mechanisms in these organisms. Furthermore, such short-term experiments designed from this novel host-pathogen model will be further applied to examine the response of clams in various environmental conditions and consequently, to detect possible disturbance of their immune system.

Experimental metal contamination

Oysters, *C. gigas*, were exposed in the laboratory to sublethal concentrations of cadmium ions. The higher doses (0.3 and 0.5 ppm) induced acute hemocytosis after 7 days (Table 2).

Differential counts in hemocyte monolayers showed that all three concentrations had the comparable effect of increasing the number of small cells within the hyalinocyte subpopulation (cells with only a narrow ring of cytoplasm around a large nucleus), and decreasing the number of large cells (Figure 6). The total number of hyalinocytes was not affected by exposure, nor was the number of

Table 2
Total hemocyte counts in oysters *Crassostrea gigas* exposed to cadmium ions ($CdCl_2$).

	0.1 ppm	0.3 ppm	0.5 ppm
Day 0	3.81 (0.77)	3.81 (0.77)	3.81 (0.77)
Day 1	2.80 (0.32)	6.88 (0.92)	4.53 (0.50)
Day 3	3.85 (0.55)	7.36 (0.53)	6.03 (0.82)
Day 7	3.61 (0.38)	7.18 (0.73)	7.68 (0.90)

Values represent mean number (10^6 cells/mL, n = 15 individuals in each sample). Numbers within parentheses are the standard error.

granulocytes. Only the number of large vacuolized cells increased from day 1 to day 7. This effect was also induced by the higher dose but, in this case, occurred earlier.

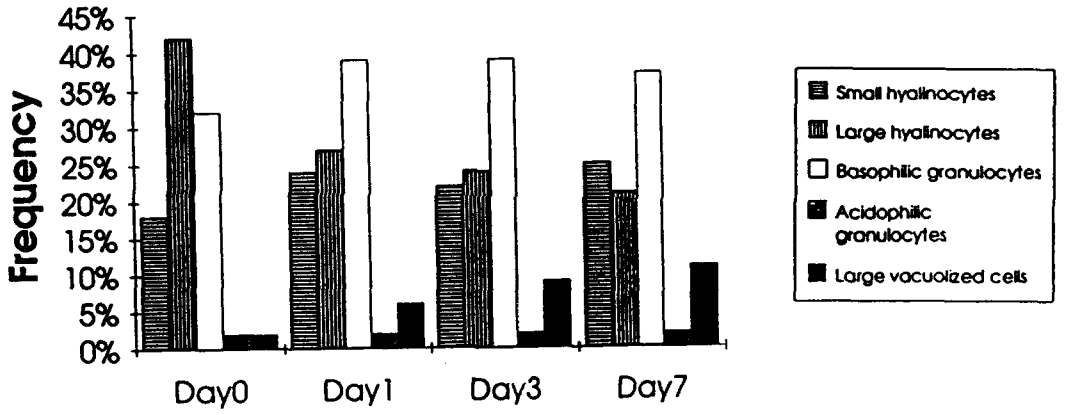

Figure 6. Differential counts of *C. gigas* hemocytes after exposure to 0.3 ppm cadmium ions ($CdCl_2$). Counts were made in cytocentrifuged monolayers of circulating cells, obtained from the adductor muscle and stained with May-Grunwald-Giemsa. Values are expressed as frequency of each cell type (n>200 in 3 oysters per sample).

Since the main overall subpopulation proportions did not change, each of them may have contributed to the observed hemocytosis. The mechanisms responsible for hemocytosis are not known but migration from the tissues into the circulating compartment is a possibility. After exposure of American oysters, *C. virginica*, to cadmium, Cheng (1988) found an increased number of cells with reduced, non-granular cytoplasm that could correspond to the small hyalinocytes we have observed in *C. gigas*. Sami *et al.* (1992) measured by flow cytometry increased numbers of small cells in oysters exposed to hydrocarbons. These similar observations of increased numbers of undifferentiated cells in the circulating compartment suggests that an effect of cadmium ions would be to induce the release of quiescent or bound hemocytes from tissues. Elevated granulocyte numbers have been reported after exposure of oysters to copper (Ruddell and Rains, 1975). More recent observations by Suresh and Mohandas (1990) of a depression in the total number of circulating cells in clams exposed to copper. This depression may have been the result of environmental interference with metal uptake by the clams, but the possibility is not excluded that copper which could have

different biotoxic effects (Pickwell and Steinert, 1984). Nevertheless, hemocytosis induced by contaminant exposure indicates some alteration in hemolymph and consequently, in the immune system. Recent observations by Anderson *et al.* (1992) of a dose-dependent suppression of chemiluminescence in oyster hemocytes exposed *in vitro* to cadmium ions supports this idea and underlines the need for complementary experiments to determine the nature of this disturbance in phagocytic cell activity and other nonspecific immune mechanisms.

CONCLUSION

Even if great variations in individual numbers have been reported in previous studies, total circulating cell counts constitute the first, easy-to-access cytometric parameter that can be applied to homogenous, population samples.

It is important to realize that all of the hemocytes are not found in the systemic circulation, since these organisms have open circulatory systems. This particular organization gives rise to the possibility of cell migration from tissues to the circulatory compartment or visa versa. Changes in cytometric parameters described here have been interpreted as a response of the immune system, a physiological response, or a direct alteration resulting from contaminant exposure. In every case, the ability to produce the cellular response may be affected, leading to increased susceptibility to pathogens. Responses observed after experimental pathogen challenge, offer interesting parallels for analyzing the effects of environmental factors on the immune system of these organisms.

Little is known on hemocytopoiesis in bivalve molluscs. As discussed above, changes in total hemocyte counts may not necessarily reflect involvement of cell production. However, since their internal defense is thought to mainly depend upon cellular mechanisms, any severe decrease in the number of circulating cells may be considered as a state of immunosuppression.

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Chapter 4

Immunotoxicology of an Anadromous Fish: Field and Laboratory Studies of B-Cell Mediated Immunity

M. R. Arkoosh, J. E. Stein, and E. Casillas

Environmental Conservation Division
Northwest Fisheries Science Center
National Marine Fisheries Service
National Oceanic & Atmospheric Administration
2725 Montlake Boulevard East
Seattle, Washington 98112

ABSTRACT

Immune function was assessed in juvenile chinook salmon from Puget Sound, WA, that were exposed to contaminants in both the field and laboratory. In the field study, salmon were collected from a contaminated urban estuary, the Duwamish Waterway estuary, as well as from a minimally contaminated nonurban estuary, the Nisqually River estuary, to determine if exposure to toxic chemicals affects their ability to produce a B-cell mediated immune response. Juvenile chinook salmon were also sampled from the two hatcheries that release salmon into these estuaries. Exposure of salmon from the Duwamish Waterway to contaminants was confirmed by increased levels of biliary fluorescent aromatic compounds, which is indicative of aromatic hydrocarbon exposure, and increased levels of polychlorinated biphenyls (PCBs) and DNA adduct formation in liver compared to salmon from the Nisqually River estuary or the hatcheries. Functional assessment of B-cell mediated immunity was determined by the plaque assay. Primed anterior kidney (AK) leukocytes from salmon collected from the urban estuary were the only cells unable to produce a heightened *in vitro* secondary plaque-forming (PFC) response to the T-dependent (T-D) antigen, trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH). Anterior kidney leukocytes from primed salmon collected from all four areas were able to produce a heightened secondary PFC response to the T-independent (T-I) antigen, trinitrophenyl-lipopolysaccharide (TNP-LPS). However, the secondary PFC response generated in primed chinook salmon collected from the urban estuary to the T-I antigen was significantly lower than that produced in salmon from the hatchery. This suppressed secondary PFC response in primed AK cells to TNP-LPS was not observed in salmon collected from the nonurban estuary and its hatchery. To confirm that the PFC assay can quantitate immune dysfunction in salmon exposed to contaminants, laboratory studies were conducted whereby juvenile chinook salmon were injected intraperitoneally with either 7,12-dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon, or with Aroclor 1254, a commercial mixture of PCBs. Both of these contaminants have been characterized as immunosuppressive in other animals and are representative of toxiants in the Duwamish Waterway. In general, the secondary PFC response of both primed AK and splenic (SP) derived B-cells to both TNP-LPS and TNP-KLH in

salmon exposed to either DMBA or PCBs was suppressed. Suppression of the primary PFC response to TNP-LPS was also observed, although suppression was dependent on leukocyte origin and was contaminant specific. The results of the laboratory study suggest that immunosuppression observed in juvenile chinook salmon from the contaminated estuary may be due to chemical contaminants. Both the laboratory and field studies suggest that the mechanism involved in generating immunological memory is more sensitive to toxicant exposure than the mechanism involved in generating a primary response to TNP-KLH and TNP-LPS. It would also appear that the mechanism involved in generating memory to a T-dependent antigen is more sensitive to toxicant exposure than to a T-independent antigen. Insight from these toxicant studies will be used to discuss how fish may generate a primary and secondary PFC response to TNP-LPS and TNP-KLH and how toxicants might affect these responses.

INTRODUCTION

Immune dysfunction in mammals has recently been recognized as a sublethal effect of xenobiotic exposure affecting both cellular and humoral aspects of the immune system (Dean *et al.*, 1986). A recent study by McCain *et al.* (1990) demonstrated that juvenile fall chinook salmon (*Oncorhynchus tshawytscha*), which migrate through a contaminated urban estuary, bioaccumulate polychlorinated biphenyls (PCBs) in the liver and have increased levels of compounds indicative of exposure to polycyclic aromatic hydrocarbons (PAHs) in the gall bladder compared to salmon that migrate through a minimally contaminated nonurban estuary. Halogenated aromatic hydrocarbons and PAHs, are known immunosuppressive agents in both mammals (Vos 1977; Koller and Thigpen, 1973; Dean *et al.*, 1986; Dean *et al.*, 1990) and fish (Spitsbergen *et al.*, 1986; Payne and Fancey, 1989). It is unknown, however, whether the levels of contaminants to which juvenile salmon are exposed, during their transit through a contaminated estuary, are sufficiently high to induce immune dysfunction.

In this present study, juvenile chinook salmon were collected from both a contaminated urban and a minimally contaminated nonurban estuary a minimum of two weeks after the initial hatchery release as described by McCain *et al.* (1990) and exposure to PCBs and PAHs assessed. Salmon were also collected from the hatchery systems just prior to their release into the estuaries. Exposure to PCBs was determined by measuring concentrations in liver, while exposure to PAHs, which are extensively metabolized by fish (Varanasi *et al.*, 1989a), was determined by measuring the concentrations of fluorescent aromatic compounds (FACs) in bile and level of binding of hydrophobic aromatic compounds to DNA. Previous studies with fish from areas contaminated with polycyclic aromatic compounds (PACs) have shown that several of the FACs detected in bile are metabolites of PAH (Krahn *et al.*, 1987) and that hepatic DNA adducts detected by ^{32}P-postlabeling appear to be due to, in large part, binding of PACs to DNA (Varanasi *et al.*, 1989b, Stein *et al.*, 1992). B-cell mediated immunity of these animals was studied by analyzing the functional ability of leukocytes from the anterior kidney and spleen, to mount an *in vitro* primary and secondary immune response to a T-dependent (T-D) antigen, trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), or to a T-independent (T-I) antigen, TNP-lipopolysaccharide (TNP-LPS; Arkoosh and Kaattari 1991).

Although we demonstrated that salmon exposed to toxicants as they transverse an urban estuary have an altered PFC response, it was unknown if individual compounds or known mixtures of contaminants can effectively suppress the salmon's B-cell mediated immunity and if this suppression can be adequately characterized by the plaque-forming cell (PFC) assay. Therefore, in the

laboratory study, we demonstrated that the PFC assay can adequately and sensitively characterize immune dysfunction in leukocytes from the anterior kidney and spleen of juvenile salmon exposed to known murine immunosuppressants (Ward *et al.*, 1985; Dean *et al.*, 1990). The model compounds, the PCB mixture, Aroclor 1254 and the PAH, 7, 12-dimethylbenz[a]anthracene (DMBA), were chosen for this study not only for their known ability to cause immunosuppression in other species but also because they represent classes of contaminants that juvenile salmon are exposed to in urban environments.

MATERIALS AND METHODS

FIELD STUDY

Salmon collection

Juvenile fall chinook salmon were collected from the Nisqually System and the Green-Duwamish system in 1990. Chinook salmon from the Kalama Creek Hatchery are released into the Nisqually River estuary which is surrounded by a predominantly rural area (Figure 1). The Nisqually System is comprised of salmon sampled from the Nisqually River estuary and the Kalama Creek Hatchery. The Nisqually River estuary enters Puget Sound about 80 km south of Seattle, WA, and is adjacent to a predominately rural area. Chinook salmon, from the Green River Salmon Hatchery, are released into the Green-Duwamish River system. The Green-Duwamish System is comprised of salmon sampled from the Duwamish Waterway estuary and the Green River Salmon Hatchery. The Duwamish Waterway estuary is surrounded by industrial sections of Seattle and enters Puget Sound through Elliott Bay (Figure 1). Sections of this urban estuary are routinely dredged and fifteen small streams and at least 20 storm drains flow into the system (McCain *et al.*, 1990).

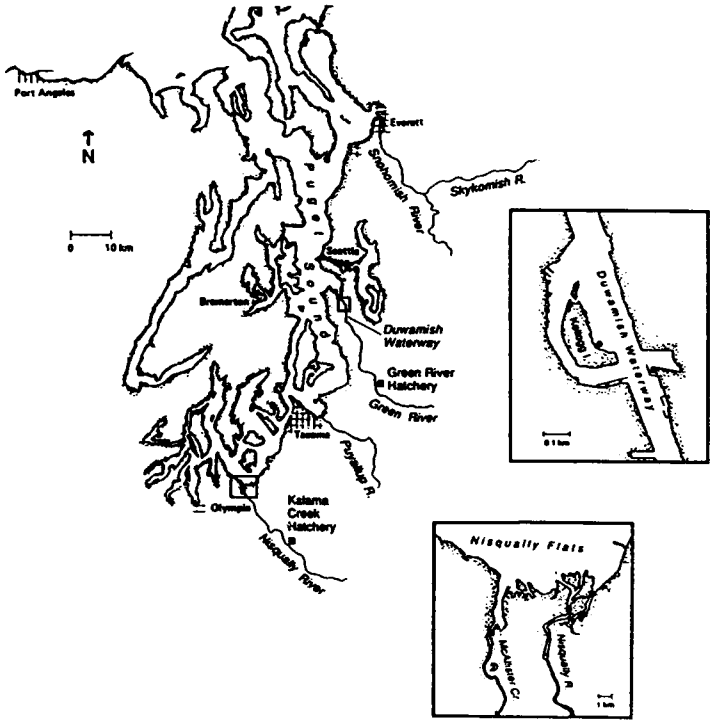

Figure 1. Map of the Puget Sound area. The dot (●) represents the sampling sites for collecting juvenile fall chinook salmon (*Oncorhynchus tshawytscha*).

Three hundred fall chinook salmon smolts were collected during May from both the Green River Hatchery and the Kalama Creek Hatchery and transported in aerated tanks filled with water from the hatchery to the National Marine Fisheries Service Field Station, located at Mukilteo, WA. The juvenile salmon were slowly acclimated from fresh water to sea water over a five day period. Outmigrating juvenile fall chinook salmon were collected from the Duwamish Waterway estuary during May and June and from the Nisqually River estuary during June with a 30 m beach seine. The salmon collected from these estuaries were transported in aerated tanks filled with estuarine water to the Mukilteo Field Station. Salmon from the estuaries were acclimated to full strength sea water over a three day period. Juvenile salmon were allowed to adapt at the Mukilteo Field Station for a minimum two week period prior to the beginning of the immunological tests.

The juvenile salmon were fed 3 % of their body weight daily with Oregon Moist Pellet (OMP; Moore-Clark, LaConner, WA)* for 10 days and alternatively for 10 days with OMP (2% of their body weight) supplemented with oxytetracycline (4 g/100 g of diet) at 1 % of their body weight.

Biomarkers of exposure (chemical and biochemical)

Liver and bile samples were collected from the salmon immediately after capture for the determination of contaminant exposure. Salmon from both systems were sampled for determining the concentrations of hepatic DNA adducts (Stein *et al.*, 1992) PCBs (Krahn *et al.*, 1988) and biliary FACs (Krahn *et al.*, 1986). Biliary FACs are a semiquantitative estimate of the concentration of polycyclic aromatic compounds, including PAH metabolites, in bile. Hepatic DNA adducts were determined by ^{32}P-postlabeling and represent binding of hydrophobic aromatic compounds, such as PAHs, to DNA.

Immunization

Both anesthetized estuary and hatchery chinook salmon were injected intraperitoneally (*i.p.*) with either 100 µL of 1 mg TNP-KLH/mL phosphate buffered saline (PBS) or with 100 µL of PBS emulsified in an equal volume of Freund's Complete Adjuvant (FCA) at least 2 weeks after they were brought to the Mukilteo Field Station. Salmon injected with antigen will be referred to as primed while those injected with PBS will be referred to as unprimed. Salmon from both the hatchery and estuary of a particular system were immunized at the same time (Arkoosh *et al.*, 1991).

Leukocyte culturing and plaque assay

Both primed and unprimed fish were netted from their tanks after immobilization with MS-222 at 9 weeks post primary injection for the Nisqually System and at 12 weeks for the Green-Duwamish System. Ten salmon were sampled from each of the estuaries and hatcheries. The ability of anterior kidney leukocytes from unprimed and primed salmon to produce a primary and secondary *in vitro* PFC response, respectively, was ascertained by exposure to the T-I antigen, TNP-LPS, or with a T-D antigen, TNP-KLH (Arkoosh and Kaattari, 1991). Leukocytes from the spleen were exposed *in vitro* only to TNP-LPS and not to TNP-KLH due to the limited number of cells available in a juvenile chinook salmon's spleen. A plaque assay was performed to enumerate the number of B cells producing anti-TNP antibodies (Arkoosh *et al.*, 1991).

Statistical analyses

Differences among means were determined by analysis of variance (ANOVA). The ANOVA was performed using the Statview™ computer program (Feldman and Gagon, 1986). Statistical significance was set at a ≤ 0.05.

LABORATORY STUDY

Salmon collection

Twenty-five hundred fall juvenile chinook salmon were collected from the Green River Hatchery in 1992. The salmon were transported in aerated tanks to the Mukilteo Field Station. Juvenile chinook salmon were acclimated from fresh water to sea water and fed 2% of their body weight daily with OMP supplemented with oxytetracycline (4 g/100 g of diet) at 1% of their body weight.

Chemicals

Stock solutions of DMBA (Sigma Chemical Co.) and Aroclor 1254 (Mansanto Chemical) were placed in a 1:1 mixture of Emulphor EL-620 (GAF Corporation) and acetone (Mallinckrodt Specialty Chemicals Co.). Various concentrations of these xenobiotics, for the determination of the median lethal dose (LD_{50}), were derived from these stock concentrations.

Median Lethal Dose (LD_{50}) of Aroclor 1254 and 7, 12-Dimethylbenz[a]anthracene

After the salmon were acclimatized for 2 weeks at the sea water facility, 96 hr LD_{50} bioassays were conducted. The juvenile chinook salmon were injected *i.p.* with a constant volume (3 mL/kg) of either Aroclor 1254 or with DMBA. The final dosages of PCBs were 1, 10, 50, 100, 250, and 500 mg/kg body weight and the final DMBA dosages were 15, 25, 35, 50, 65, 75, and 88 mg/kg. Controls were injected with a constant volume (3 mL/kg) of the acetone/Emulphor EL-620 vehicle. Thirty salmon were injected per concentration. Mortalities were tabulated every 24 hr for 96 hr from the time of injection.

Contaminant Exposure and Immunization

Groups of 200 anesthetized (1.5 mg/L of metomidate) juvenile chinook salmon were injected *i.p.* with a constant volume (3 mL/kg of fish) of either acetone:Emulphor EL-620 (50:50) as controls, 20% of the Aroclor 1254 or DMBA 96 hr LD_{50} or 1% of the DMBA 96 hr LD_{50}. One week after injection with xenobiotics, 100 salmon from each of the four groups were injected *i.p.* with 100 µL of a 1 mg/mL solution of TNP-KLH solution emulsified in an equal volume of FCA. The remaining 100 fish from each treatment were injected with 100 µL of PBS emulsified in an equal volume of FCA. Salmon injected with antigen will be referred to as primed while those injected with PBS will be referred to as unprimed.

Leukocyte culture and plaque assay

Both primed and unprimed fish were netted from their tanks after immobilization with metomidate at 9 weeks post primary injection. The *in vitro* primary and secondary PFC response of anterior

kidney and splenic leukocytes from individual salmon to TNP-KLH and TNP-LPS was determined as described for the field study (Arkoosh *et al.*, 1991).

Statistical analyses

The differences among means were determined by ANOVA which was performed using the Statview™ computer program (Feldman and Gagon, 1986) and the 96 hr LD_{50}s for the two xenobiotics were determine by the logit method using DeltaGraph™ (Wulf *et al.*, 1991). Statistical significance was set at $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

FIELD STUDY

Juvenile salmon from the Duwamish Waterway estuary had significantly higher levels of hepatic DNA adducts and biliary FACs, which are both indicative of exposure to PACs, and liver PCBs than did the salmon from the hatcheries or the Nisqually River estuary (Figure 2). These findings show that the salmon from the Duwamish Waterway estuary were exposed to significantly higher levels of PCBs and PACs, including PAHs, than salmon from the hatcheries or the Nisqually River estuary (Stein *et al.*, in prep).

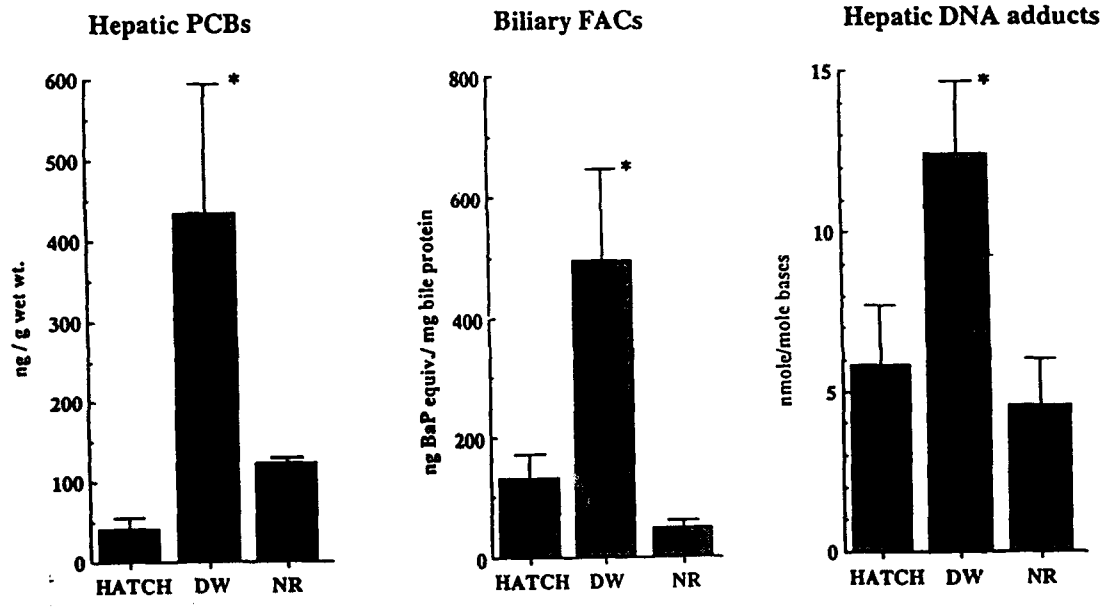

Figure 2. Levels of hepatic PCBs, biliary FACs and hepatic DNA adducts. The asterisk (*) signifies estuary salmon with significantly higher levels of the particular biomarker than the amount determined in hatchery fish. Data obtained from Hatchery fish (Green River and Kalama Creek Hatchery) were combined for the above the above comparisons since they were not significantly different. The abbreviation (DW) signifies data determined for salmon from the Duwamish Waterway estuary. The abbreviation (NR) signifies data determined for salmon from the Nisqually River estuary (Stein *et al.*, in prep).

Leukocytes from the anterior kidney of primed juvenile salmon collected from the Green River Hatchery, Kalama Creek Hatchery and the Nisqually River estuary were able to generate a significantly higher secondary PFC response to TNP-KLH (Figure 3) than that produced during the primary PFC response of unprimed salmon from these areas. However, this heightened secondary PFC response to TNP-KLH did not occur with leukocytes from the anterior kidney of primed Duwamish Waterway estuary juvenile salmon (Figure 3).

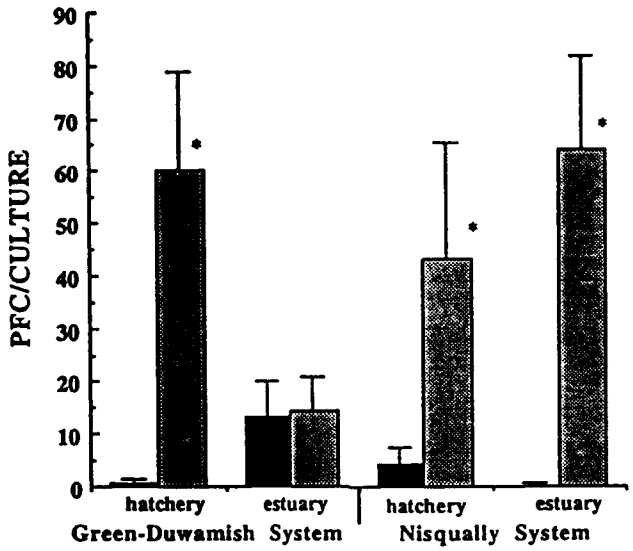

Figure 3. The primary (■) and secondary (■) *in vitro* plaque forming cell response per culture (PFC/culture) to TNP-KLH generated in the leukocytes from the anterior kidney. The mean (±SD) PFC response was analyzed in chinook salmon from the Green-Duwamish System and the Nisqually System. Anterior kidneys from 10 chinook salmon were examined from each of the four locations. Anterior kidney cells cultured only with tissue culture medium (background) did not produce more than a mean of 2 PFC/culture. The asterisk (*) indicates the secondary PFC/culture that is significantly higher ($p \leq 0.05$) than observed in the primary response (Arkoosh *et al.*, 1991).

The secondary PFC response of leukocytes from the anterior kidney of primed juvenile salmon to TNP-LPS was significantly higher than the primary PFC response in salmon from all four locations (Figure 4). However, the secondary PFC response generated by leukocytes from the anterior kidney of primed juvenile salmon from the Green River Hatchery was significantly different from that produced by primed juvenile salmon from the Duwamish Waterway estuary (Figure 4). The leukocytes from the anterior kidney of primed salmon from the Green River Hatchery produced a significantly higher ($p \leq 0.05$) secondary PFC response than the leukocytes from the anterior kidney of primed salmon from the Duwamish Waterway. However, the secondary PFC response to TNP-LPS of leukocytes from the anterior kidney of primed salmon from the Kalama Creek Hatchery and Nisqually River estuary was not significantly different (Figure 4). Additionally, the primary PFC response of leukocytes to TNP from the anterior kidney of unprimed juvenile chinook salmon appeared not to be affected (Figure 3 & 4) by estuary exposure.

Leukocytes from the spleen of primed juvenile salmon collected from the Green-Duwamish System and the Nisqually System were able to produce a significantly higher secondary PFC response to TNP-LPS compared to the primary PFC response produced in unprimed salmon (Figure 5). The secondary PFC response to TNP-LPS of leukocytes from the spleen in primed salmon from Green River Hatchery was significantly higher ($p \leq 0.05$) than the secondary PFC response produced by

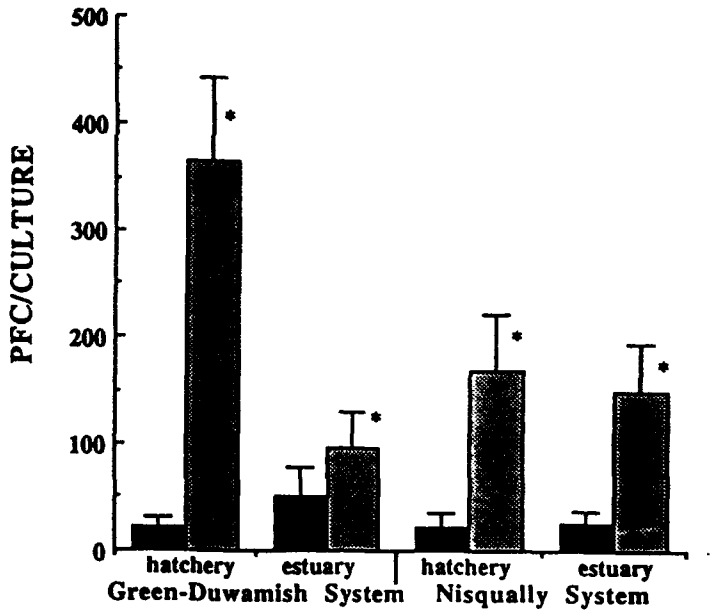

Figure 4. The primary (■) and secondary (■) *in vitro* plaque forming cell response per culture (PFC/culture) to TNP-LPS generated in leukocytes from the anterior kidney. The mean (±SD) PFC response was analyzed in chinook salmon from the Green-Duwamish System and the Nisqually System. Anterior kidneys from 10 chinook salmon were examined from each of the four locations. Anterior kidney cells cultured only with tissue culture medium (background) did not produce more than a mean of 2 PFC/culture. The asterisk (*) indicates the secondary PFC/culture that is significantly higher ($p \leq 0.05$) than observed in the primary response (Arkoosh *et al.*, 1991).

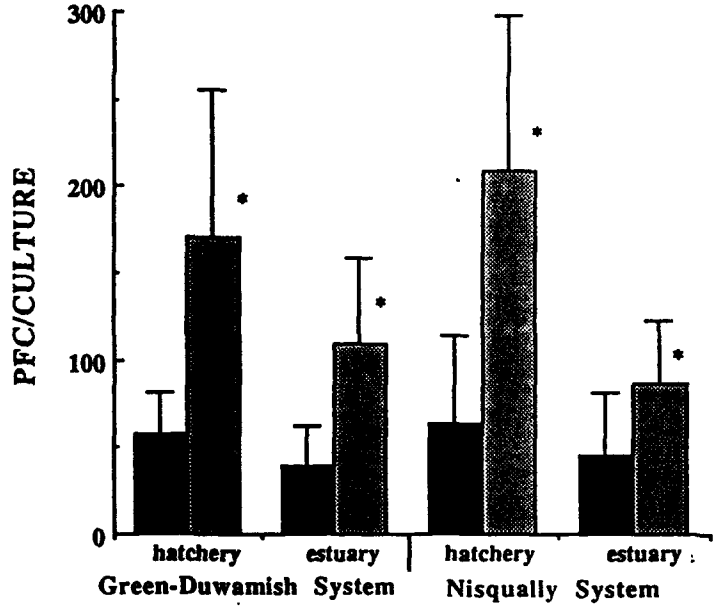

Figure 5. The primary (■) and secondary (■) *in vitro* plaque forming cell response per culture (PFC/culture) to TNP-LPS generated in leukocytes from the spleen. The mean (±SD) PFC response was analyzed in chinook salmon from the Green-Duwamish System and the Nisqually System. Spleens from 10 chinook salmon were examined from each of the four locations. Spleen cells cultured only with tissue culture medium (background) did not produce more than a mean of 4 PFC/culture. The asterisk (*) indicates the secondary PFC/culture that is significantly higher ($p \leq 0.05$) than observed in the primary response (Arkoosh *et al.*, 1991).

leukocytes from the spleen of primed Duwamish Waterway estuary fish (Figure 5). However, this same pattern was observed in juvenile chinook salmon from the Nisqually System. The secondary PFC response of leukocytes from the spleen to TNP-LPS in primed fish from the Kalama Creek Hatchery was significantly higher ($p \leq 0.05$) than the PFC response produced by the leukocytes from the spleen of primed salmon from the Nisqually River estuary (Figure 5). The primary PFC response produced in the leukocytes from the unprimed spleen of juvenile chinook salmon to TNP-LPS appeared not to be affected (Figure 5) by estuary exposure.

This is the first field study with fish to examine B-cell mediated immunity via the primary and secondary PFC response. The observed suppressed secondary *in vitro* PFC response may be linked to contamination. Salmon collected from the urban Duwamish Waterway had a significantly higher mean concentration of PCBs in the liver than that found in the juvenile chinook salmon from the nonurban Nisqually River estuary. In addition, the mean concentration of FACs in bile and hepatic DNA adduct levels were significantly higher in fish from the Duwamish Waterway than the concentrations found in the Nisqually River salmon. Salmon from the respective hatcheries, Green River Salmon Hatchery and Kalama Creek Hatchery, had similar low levels of PCBs and biliary FACs and the levels were comparable to those in salmon from the Nisqually River estuary. This suggests that the altered immune responses in salmon from the Duwamish Waterway may be due to the effects of chemical contaminants.

Although numerous abiotic and biotic factors can affect the immune system, our experimental design allowed us to differentiate those factors from the effects of contaminants on juvenile salmonids. By determining the PFC response of hatchery fish as well as estuary fish, our study normalized the unique components of each watershed (*i.e.* nutrition and temperature) that may influence the immune response. This approach allowed us to determine the normal basal PFC response of fish from a particular watershed and formed the bases of comparison for juvenile salmon from the different watersheds.

Unlike the response of leukocytes from the anterior kidney, suppression of the secondary PFC response due to toxicant exposure was not observed in leukocytes from the spleen of primed juvenile chinook salmon from an urban estuary. These results suggest a dichotomous behavior in the immunological response between the anterior kidney, a primary lymphoid organ (Kaattari and Irwin, 1985), and the spleen, a secondary lymphoid organ, to contaminant exposure and demonstrates the importance of examining both lymphoid organs in such studies. Also, as demonstrated with leukocytes from the anterior kidney of unprimed salmon, no immunomodulatory effects were observed in the primary PFC response of leukocytes from the spleen of unprimed salmon to TNP.

In summary, we have demonstrated that juvenile chinook salmon, from an urban estuary and exposed to PCBs and PAHs have a suppressed *in vitro* secondary PFC response to TNP. Although immunosuppression was observed in the anterior kidney response, suppression due to contaminant exposure was not found in the spleen.

LABORATORY STUDY

Median lethal dose (LD_{50}) of Aroclor 1254 and 7, 12-Dimethylbenz[a]anthracene (DMBA)

The 96 hr LD_{50}S were determined for salmon injected with either DMBA or PCBs to insure that their potential immunosuppressive effects would be assessed at sublethal dosages of these two

chemicals. The LD_{50} determined for Aroclor 1254 was 271 mg/kg of salmon (Figure 6) and the LD_{50} determined for DMBA was 63 mg/kg of salmon (Figure 7). Mortalities never exceeded 3% in control salmon during the 96 hr period.

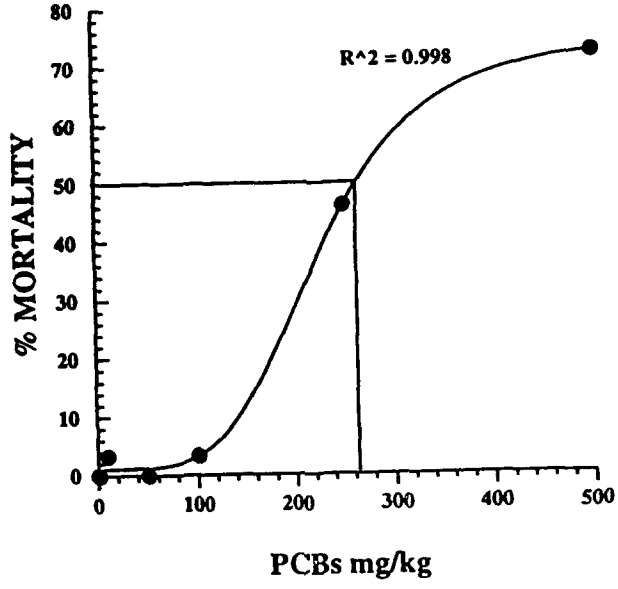

Figure 6. The cumulative 96 hr mortality of salmon given various doses of Aroclor 1254. A total of 30 juvenile chinook salmon were injected (3 mL/kg) per dose of Aroclor 1254 (Arkoosh *et al.*, in press).

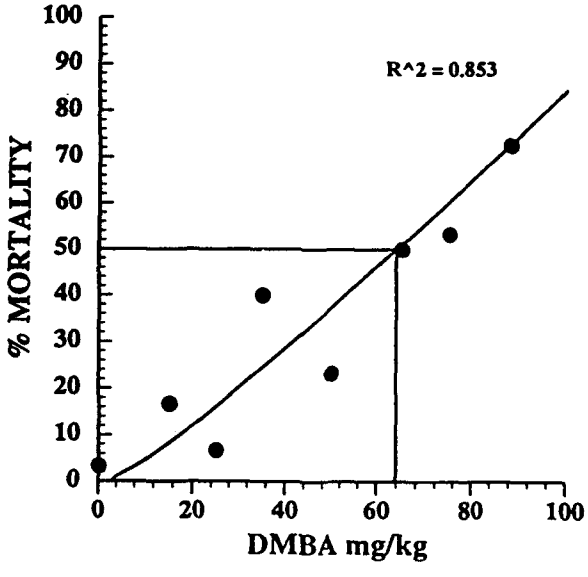

Figure 7. The cumulative 96 hr mortality of salmon given various doses of DMBA. A total of 30 juvenile chinook salmon were injected (3 mL/kg) per dose of DMBA (Arkoosh *et al.*, in press).

Immunological Effects

Juvenile chinook salmon were injected with either 54 mg of Aroclor 1254/kg of salmon (20% of the 96 hr LD_{50}), 12.7 mg of DMBA/kg of salmon (20% of the 96 hr LD_{50}) or 0.6 mg of DMBA/kg of salmon (1% of the 96 hr LD_{50}). Leukocytes from the spleen of unprimed salmon injected with either DMBA or PCBs produced a significantly lower primary PFC response to TNP-LPS (Figure 8A) than those of unprimed control fish, but there was no significant difference in their ability to produce a primary PFC response to TNP-KLH (Figure 8B). Leukocytes from the spleen of control salmon primed with antigen were able to produce an enhanced secondary PFC response to TNP-LPS (Figure 8A) and TNP-KLH (Figure 8B) relative to the primary PFC response of splenic leukocytes from unprimed control salmon. However, splenic leukocytes from primed salmon injected with either DMBA or PCBs were unable to produce an enhanced secondary *in vitro* PFC response relative to the primary PFC response from unprimed exposed salmon to either TNP-LPS or TNP-KLH (Figure 8A, B). Due to the limiting number of splenic cells available in a juvenile salmon, we were unable to examine the secondary PFC response of splenic leukocytes to TNP-KLH from salmon exposed to 12.7 mg/kg of DMBA. The two dosages of DMBA produced equivalent degrees of suppression in both the splenic primary and secondary PFC responses to TNP-LPS (Figure 8A).

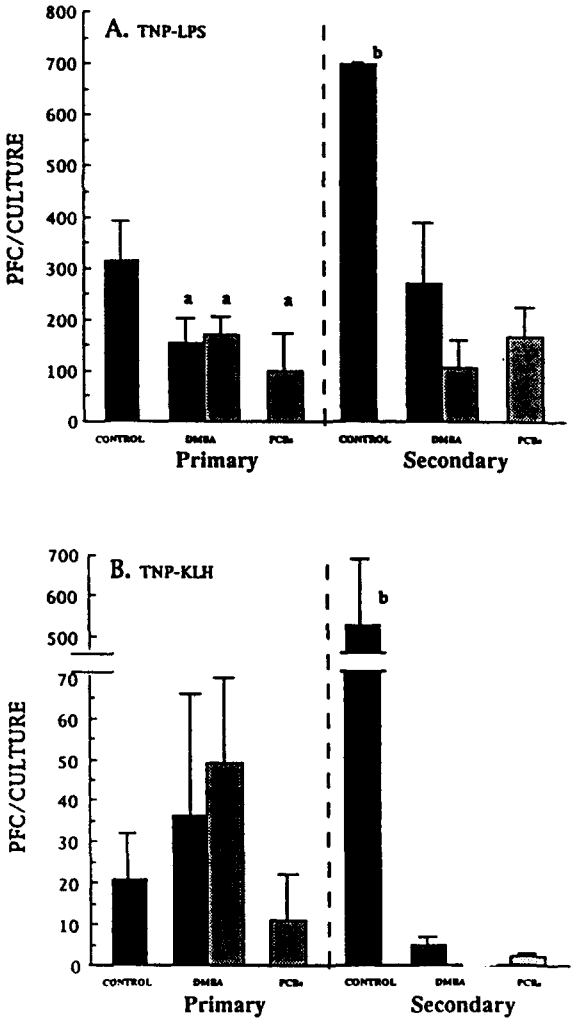

Figure 8. The mean (±SE) splenic primary and secondary *in vitro* PFC response/culture in juvenile chinook salmon exposed to DMBA at either 0.6 mg/kg of salmon (■) or at 12.7 mg/kg of salmon (■) or the PCB mixture, Aroclor 1254 (54 mg/kg of salmon) to TNP-LPS (A) and TNP-KLH (B). The (**a**) signifies that the primary PFC response/culture was significantly ($p \leq 0.05$) altered in the contaminant-exposed fish from the primary PFC response/culture observed in the Acetone/Emulphor injected controls. The (**b**) signifies an enhanced secondary PFC response/culture produced in relation to the primary PFC response/culture within the treatments. The (**c**) signifies data unavailable for secondary PFC responses/culture (Arkoosh *et al.*, in press).

Unlike splenic leukocytes, the primary anterior kidney PFC response of unprimed salmon injected with DMBA to TNP-LPS was not suppressed (Figure 9A) relative to the primary PFC response of anterior kidney leukocytes from unprimed control salmon. However, the primary anterior kidney PFC response to TNP-LPS of unprimed salmon exposed to PCBs was suppressed. As with splenic leukocytes from DMBA and PCB exposed unprimed salmon, the anterior kidney's primary PFC response to TNP-KLH was not significantly altered when compared to the primary PFC response of anterior kidney leukocytes from unprimed control salmon (Figure 9B). However, the primary anterior kidney PFC response to TNP-KLH of unprimed salmon exposed to DMBA appeared to be heightened (not significantly) when compared to the primary anterior kidney PFC response of unprimed control fish. Due to the limited number of anterior kidney leukocytes available from the unprimed and primed salmon, we were unable to examine either the primary or secondary PFC response to TNP-KLH of salmon exposed to 0.6 mg of DMBA/kg of salmon.

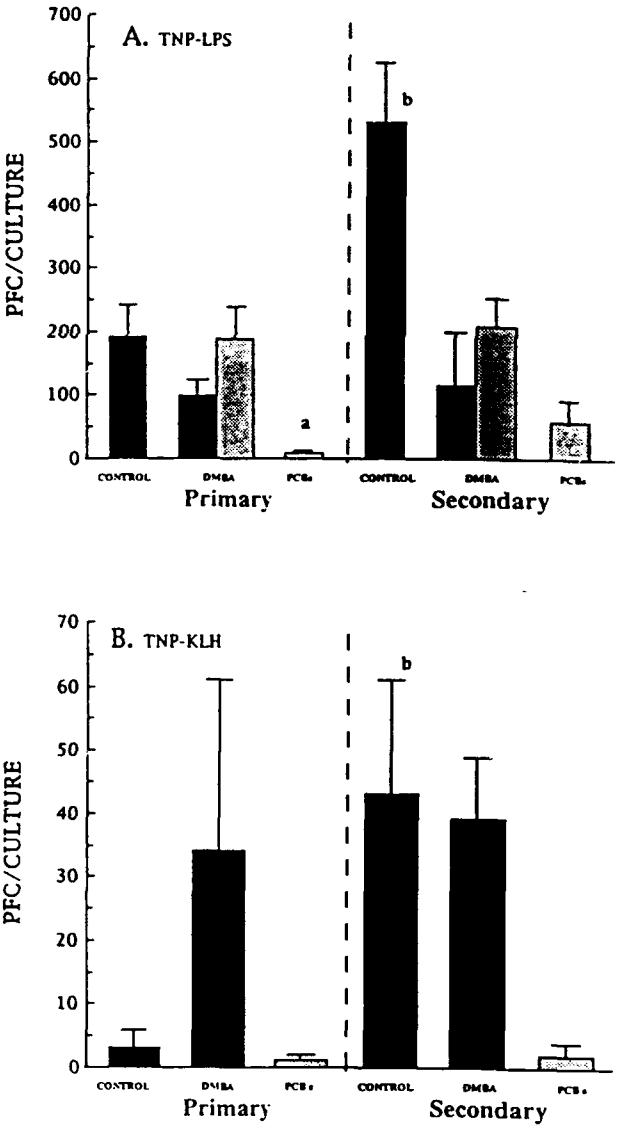

Figure 9. The mean (±SE) anterior kidney primary and secondary *in vitro* PFC response/culture of juvenile chinook salmon exposed to DMBA at either 0.6 mg/kg of salmon (■) or at 12.7 mg/kg of salmon (■) or the PCB mixture, Aroclor 1254 (54 mg/kg of salmon) to TNP-LPS (A) and TNP-KLH (B). The (a) signifies that the primary PFC response/culture was significantly ($p \leq 0.05$) altered in the contaminant-exposed fish from the primary PFC response/culture observed in the Acetone/Emulphor injected controls. The (b) signifies an enhanced secondary PFC response/culture produced in relation to the primary PFC response/culture within the treatments (Arkoosh *et al.*, in press).

Anterior kidney leukocytes from control primed salmon were able to produce an enhanced secondary PFC response to both forms of the antigen relative to the primary PFC response of unprimed control salmon (Figure 9A, B). The secondary PFC response of anterior kidney leukocytes to TNP-LPS from DMBA and PCB injected primed salmon was not enhanced over the primary PFC response observed in anterior kidney leukocytes of unprimed salmon exposed to DMBA or PCBs (Figure 9A). The two dosages of DMBA produced equivalent degrees of suppression in the secondary PFC response of anterior kidney leukocytes to TNP-LPS (Figure 9A). The secondary PFC response of anterior kidney leukocytes to TNP-KLH from DMBA and PCB injected primed salmon was not enhanced over the primary PFC response observed in anterior kidney leukocytes from unprimed salmon exposed to DMBA and PCBs, respectively (Figure 9B).

The development of immunological memory in fish as defined by an enhanced secondary PFC response relative to the primary PFC response to an antigen (Arkoosh and Kaattari, 1991), did not occur in juvenile chinook salmon exposed to PCBs or DMBA. However, whether an altered primary PFC response due to xenobiotic exposure occurred in juvenile chinook salmon depended upon the lymphoid organ examined, type of antigen used for stimulating the leukocytes, and whether the salmon were exposed to DMBA or Aroclor 1254. Therefore, as also demonstrated in the field study (Arkoosh *et al.*, 1991) examining the immune response of juvenile chinook salmon from an urban estuary, the importance of examining various lymphoid tissues, antigens, and both the primary and secondary PFC response in immunotoxicity studies is crucial in determining the immunoaltering profile of a contaminant.

We found that in juvenile chinook salmon, the secondary anterior kidney and splenic PFC response was altered and appeared to be a more sensitive indicator of immunosuppressive effects of PAH and PCB exposure than the primary anterior kidney and splenic PFC response. For example, the primary splenic PFC response to TNP-KLH in salmon exposed to PCBs was comparable to the PFC response in unprimed control fish, but the secondary PFC response to TNP-KLH was suppressed relative to the secondary PFC response in primed control salmon. We also observed suppression of the secondary PFC response to TNP-LPS in the anterior kidney leukocytes of primed juvenile chinook salmon exposed to DMBA, while the primary anterior kidney leukocyte PFC response to TNP-LPS of salmon exposed to DMBA was not statistically altered from unprimed controls.

Although the secondary PFC response was always altered by toxicant exposure, alterations in the primary PFC response depended upon the antigen being used to stimulate the leukocytes. The primary PFC response to the T-I antigen, TNP-LPS, in unprimed juvenile chinook salmon appears to be more affected by contaminant exposure than their primary PFC response to the T-D antigen, TNP-KLH. Unprimed juvenile chinook salmon exposed to either DMBA or PCBs have a suppressed primary splenic PFC response to the T-I but not to the T-D antigen. This has also been observed in rainbow trout exposed to PCBs. As in the present study, rainbow trout exposed to Aroclor 1254 did not have an altered primary splenic PFC response to a T-D antigen, SRBC (Cleland *et al.*, 1988). However, rainbow trout fed the PCB, Clophen A50 (500 mg/kg), had a significantly suppressed serum primary antibody response to *V. anguillarum* 0 antigen, a T-I antigen (Thuvander and Carlstein, 1991). Using a much lower concentration of PCB (54 mg/kg), we were also able to demonstrate a significantly suppressed primary anterior kidney and splenic PFC response to the T-I antigen, TNP-LPS.

In summary, juvenile chinook salmon exposed to the carcinogenic PAH, DMBA, or the PCB mixture, Aroclor 1254, exhibited an altered PFC response. The salmon's splenic and anterior kidney

primary PFC response to a T-I antigen appeared to be more sensitive to contaminant exposure than their primary PFC response to a T-D antigen. However, the secondary PFC response to both antigens is more likely to demonstrate a contaminant-associated effect than the primary PFC response. These studies demonstrate that the PFC assay is a promising marker of immunomodulatory effects in fish from exposure to PAHs and PCBs.

CONCLUSION

Field studies were undertaken which examined B-cell mediated immunity of juvenile chinook salmon from contaminated and minimally contaminated estuaries and from the hatcheries which release the salmon into these estuaries. B-cell mediated immunity was examined with the PFC assay. These assays were performed concomitantly with assessment of the salmon for chemical and biochemical markers of contaminant exposure. This field study was followed by a laboratory study where the salmon were exposed to two model immunomodulatory compounds: DMBA and Aroclor 1254. The functional analysis of the immune response in laboratory exposed salmon was also determined by the PFC assay.

Both the field and laboratory studies have shown that although an effect due to contaminant exposure may not occur in the primary PFC response, an effect is more likely to occur in the secondary PFC response. Deviations in the secondary PFC response occurred in both primed salmon collected from the urban estuary and injected with either DMBA or PCBs. These studies also demonstrated that the PFC assay is a sensitive indicator of contaminant exposure.

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Chapter 5

Macrophage Activation in Fish

Christopher J. Secombes

Department of Zoology
University of Aberdeen
Tillydrone Avenue
Aberdeen, AB9 2TN, UK.

INTRODUCTION

Fish macrophages can be easily isolated by a combination of density gradients and adherence to glass/plastic (Secombes, 1990). This has allowed many aspects of their biology to be examined over the last few years. Such studies have shown that fish macrophages are potent effector cells (Secombes and Fletcher, 1992), able to kill bacterial and helminth pathogens, and that they produce a number of oxygen-dependent and oxygen-independent microbicidal molecules. They can also secrete soluble mediators important in inflammatory events, including cytokines and eicosanoids. In addition, macrophages are important accessory cells for lymphocyte responses (Vallejo *et al.*, 1992), and are involved in antigen uptake and presentation. Both effector and accessory activities of macrophages can potentially be modulated in fish, with implications for eliciting protective responses against disease. Our studies on this aspect of fish macrophage biology primarily concerning rainbow trout (*Oncorhynchus mykiss*), will be reviewed here and areas requiring further investigation in fish in general will be highlighted.

In order to investigate macrophage activation it is necessary to study a number of biologically relevant assays. Probably the most important of these are assays which examine macrophage killing activity, since it is this function which determines whether a cell is activated, primed or resident (Nathan, 1986). Primed cells are usually considered to be macrophages which have some functions increased (such as phagocytosis or chemotaxis), but which have not acquired a heightened killing activity. The generation of microbicidal products is also a good choice for monitoring whether a macrophage is activated, since clearly they contribute directly to any increased killing. Such products include reactive oxygen species (ROS) produced during phagocytosis, in a process called the respiratory burst (Secombes and Fletcher, 1992). Individual ROS released following macrophage stimulation, such as superoxide anion (Secombes *et al.*, 1988) and hydrogen peroxide (Chung and Secombes, 1988), can be easily detected using a number of assays, as can the light or chemiluminescence associated with release of singlet oxygen. It is also possible to monitor the increased uptake of oxygen during this reaction (Nagelkerke *et al.*, 1990). By analogy with mammalian macrophages, the production of cytokines would similarly be interesting to monitor, but awaits the availability of purified fish reagents and specific antisera.

MACROPHAGE ACTIVATION *IN VIVO*

It is already well established that macrophage effector functions can be up-regulated *in vivo* (Secombes and Fletcher, 1992). For example, intraperitoneal injection with formalin killed bacteria (*Aeromonas salmonicida*), followed 10 days later with a booster injection elicits activated macrophages with enhanced bactericidal activity (Chung and Secombes, 1987). In addition, such cells have enhanced respiratory burst activity, acid phosphatase activity and RNA synthesis. Using the same injection regime, sonicated parasite larvae (from the Digenean *Diplostomum spathaceum*) can also generate macrophages with increased respiratory burst activity (Whyte *et al.*, 1990). Similarly, work carried out in collaboration with Dr. Børre Robertson, at the University of Tromsø, has shown that intraperitoneal injection of trout with glucan increases head kidney macrophage bactericidal and respiratory burst activity two to three weeks post-injection (Jørgensen *et al.*, 1993). Injection with glucan has also been shown to increase disease resistance non-specifically (Røbertsen *et al.*, 1990), but as several innate defences are increased it is difficult to be sure about the relative importance of macrophages in protection.

Standard dietary ingredients also influence macrophage functions, and studies in this laboratory have concentrated on the effects of nutritional lipids in collaboration with Dr. Andrew Rowley, at Swansea. Lipids are particularly important with respect to eicosanoid production by macrophages, which are potent mediators of inflammation. Eicosanoids are derived from membrane associated eicosapolyenoic acids (such as arachidonic acid) via the action of cyclooxygenase or lipoxygenase enzymes (Pettitt *et al.*, 1991). Lipoxygenase products such as leukotrienes and lipoxins shown to be released from trout macrophages (Pettitt *et al.*, 1989; Pettitt *et al.*, 1991), are potent chemoattractants for fish leukocytes (Sharp *et al.*, 1992) and influence T cell responses (Secombes *et al.*, in press). Amongst the major lipoxygenase products released by macrophages from fish fed standard diets rich in n-6 polyunsaturated fatty acids (PUFA), are leukotriene B_4 and lipoxin A_4. However, if fish are fed a diet rich in n-3 PUFA, as in fish oil rich diets, leukotriene B_5 and lipoxin A_5 predominate (Figure 1). The biological effects of the shift in eicosanoid class have still to be determined fully, although there does appear to be an effect upon phagocyte migration (Ashton *et al.*, submitted). Since T cell responses are influenced by eicosanoids, the possibility also exists that cytokine-induced activation of phagocytes will prove to be affected. Other dietary ingredients that we have examined to date, namely vitamins E, C and A, have shown little effect upon macrophage bactericidal mechanisms or pathways involved in their up-regulation (Hardie *et al.*, 1990, 1991; Thompson *et al.*, in press), despite an impact on disease susceptibility. However, addition of vitamin C (10^{-5} and 10^{-6} M sodium ascorbate) to head kidney leukocytes recovered from vitamin C depleted trout significantly increases macrophage activating factor production (Hardie *et al.*, 1993). This suggests that further studies on modulation of phagocyte activation *in vivo* by vitamins are warranted.

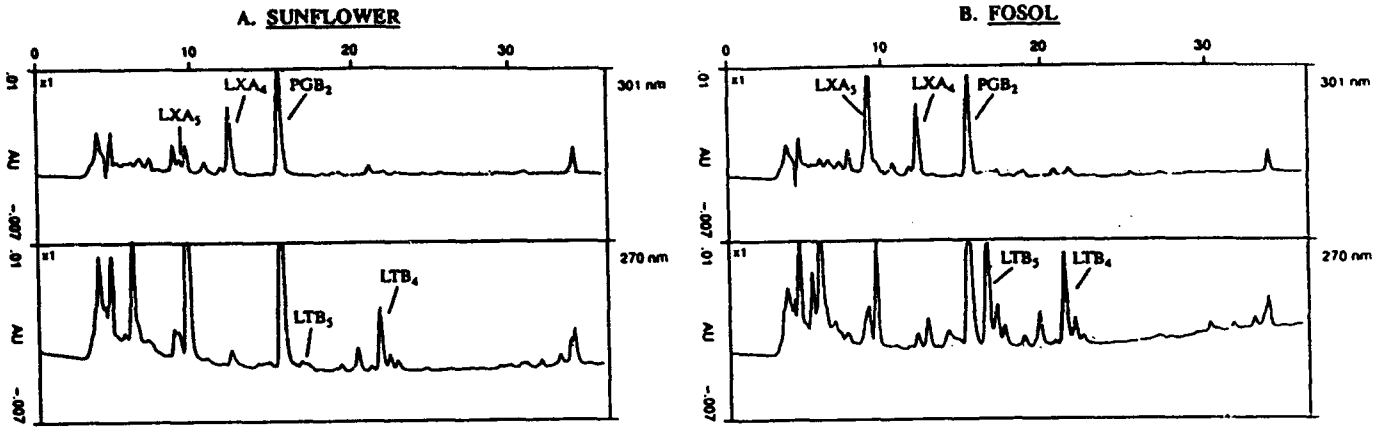

Figure 1. Representative RP-HPLC chromatograms of extracted metabolites from calcium ionophore challenged head kidney leukocytes isolated from a rainbow trout fed either a sunflower oil (A) or a fish oil (B) containing diet. Note the shift in eicosanoids from predominantly 4-series products with sunflower oil diets to a situation where 5-series products predominate with fish oil diets. LX = lipoxin, LT = leukotriene, PG = prostaglandin.

Macrophage activation *in vitro*

It is possible to modulate macrophage activity *in vitro*. In our early studies an increase in respiratory burst activity was induced by incubation of kidney macrophages with peritoneal washes from fish injected to obtain activated peritoneal macrophages (Chung and Secombes, 1987). The lack of antibody in such washes suggested this was a cytokine-mediated phenomenon. Subsequent experiments used supernatants from Con A-stimulated head kidney or peripheral blood leukocytes (Graham and Secombes, 1988), and showed they contained a factor(s) capable of inducing macrophage activation. Isolation of the macrophage activating factor (MAF) in these supernatants has proved to be difficult but it appears that it is a T (surface Ig^-) cell product (Graham and Secombes, 1990a) with a molecular weight of approximately 19kDa, and is temperature and pH sensitive. The physicochemical characteristics of this molecule combined with its coelution with interferon activity present in the same supernatants, suggest fish possess a molecule equivalent to γ-interferon (Graham and Secombes, 1990b). However, mammalian γ-interferons have no effect upon trout macrophages (personal observation).

More recently we have been able to demonstrate MAF release from mitogen-stimulated gut leukocytes (Davidson *et al.*, 1991) and from antigen-stimulated, primed head kidney leukocytes (Marsden *et al.*, in press). In this latter study the kinetics of MAF-release post-immunization with *Aeromonas salmonicida* was examined (Figure 2). MAF release was detectable from stimulated cells 3-weeks post-immunization and peaked between 4 and 5 weeks later. Specific antibody production had similar kinetics, demonstrating that specific humoral and cell-mediated immunity

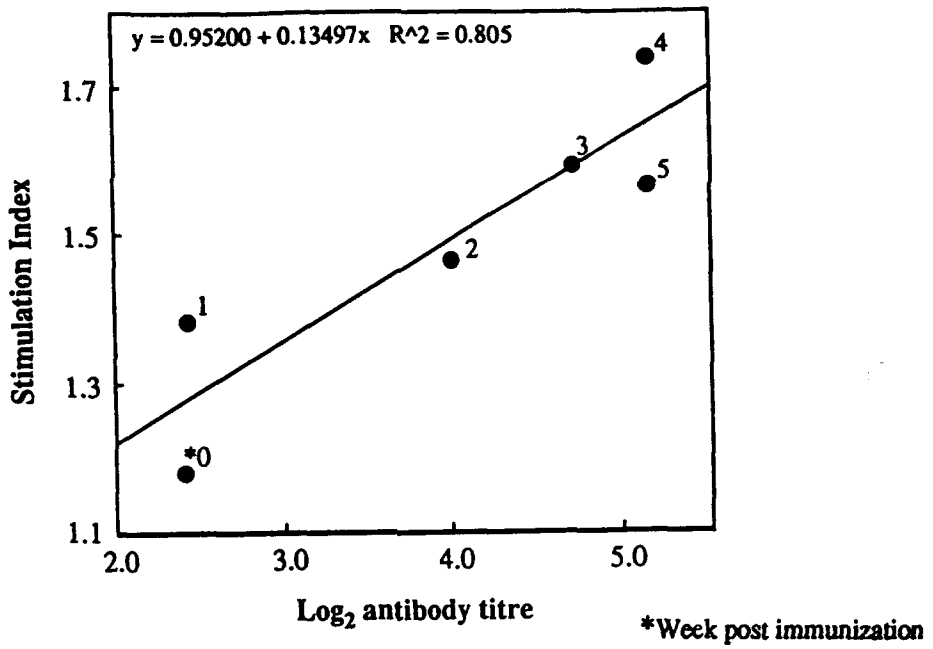

Figure 2. MAF activity versus antibody titer in rainbow trout immunized with *Aeromonas salmonicida*, over a five week period. MAF activity was expressed as a stimulation index, by dividing the respiratory burst activity of macrophages incubated with supernatants from antigen-stimulated leukocytes with that from macrophages incubated with control supernatants. There is a significant correlation between mean MAF activity and mean antibody titre. n=5 fish for each point.

can co-exist. Both cellular (whole bacteria) and soluble (extracellular products) antigens were found to stimulate MAF release from primed cells *in vitro*, although whole cells usually gave the best result.

From studies on *in vitro* activation of macrophages it is clear that many functions are increased in such cells (Secombes and Fletcher, 1992). These include their ability to adhere and spread, to undergo a respiratory burst and to phagocytose. Their lysosomal enzyme activity, as well as killing activity are also enhanced. In some cases, as with larvacidal activity, antibody is also required to enhance killing (Whyte *et al.*, 1989), and studies on erythrophagocytosis suggest activated macrophages have an increased expression/activity of Fc receptors (Secombes and Fletcher, 1992). Not all activities increase in activated macrophages and an example of an activity which decreases is 5'nucleotidase activity. MAF treatment of macrophages reveals an inverse relationship between respiratory burst activity and 5'nucleotidase activity (Hepkema and Secombes, in press), in agreement with earlier *in vivo* studies (Zelikoff and Enane, 1992). MAF-treated cells were also found to lose 5'nucleotidase activity faster than control cells in the presence of cycloheximide, suggesting such cells may have a higher membrane turnover of this ectoenzyme.

Signal synergism in macrophage activation

Although this area remains virtually unresearched in fish cells, it is possible that different signals act in synergy to enhance macrophage activity. Recently, however, we have shown that MAF-containing supernatants and human rTNFα can act synergistically to elevate respiratory burst activity

(Hardie *et al.*, 1994). In mammals, many such interactions are known to exist, giving a complex model of macrophage activation in response to combinations of inductive signals (Adams & Hamilton, 1992). The action of a mammalian cytokine (TNFα) on fish cells implies conservation of a receptor (TNF-R) that recognizes TNF. In mammals two main types of TNF-R have been cloned, TNF-R1 (55 kD) and TNF-R2 (75 kD) (Tartaglia and Goeddel, 1992), and have some 62% - 64% overall identity between species. However, the homologies are distributed differently, such that TNF-R1 is predominantly conserved within the extracellular domain whereas TNF-R2 is conserved across the intracellular domain (73%), and this appears to correlate with a lack of species-specificity of the former (Lewis *et al.*, 1991). In support of the above conclusion, using a battery of 6 anti-human TNF-R1 monoclonal antibodies (generously donated by Celltech Ltd, Slough, UK) 2 have been found to inhibit the ability of fish macrophages to respond to human rTNFα (Jang *et al.*, submitted).

The intracellular events that occur during activation of fish macrophages are unknown. Whilst undoubtedly some progress can be made in this area with respect to second messengers, macrophage activation is largely a consequence of alterations in expression of specific genes (Adams and Hamilton, 1992). Studies on the ability of signals to turn genes on or off are clearly dependent upon sequence data for relevant genes, few of which are available at present; class II MHC genes being a notable exception (Juul-Madsen *et al.*, 1992; Ono *et al.*, 1993).

Effects of suppressive signals on macrophage activation

Suppression of phagocyte functions by particular signals can also occur, and is well known in fish. For example, pollutants, autonomic neurotransmitters, stress and low temperatures all have inhibitory effects on various phagocyte functions (Secombes and Fletcher, 1992). One question that arises from these studies is whether macrophages can be activated despite the presence of potentially suppressive signals. Our studies on dab exposed to sewage sludge (Secombes *et al.*, 1992) are interesting with respect to this. An eleven week (chronic) exposure to a nominal 24 hr concentration of 0.032% sludge results in decreased head kidney leukocyte bactericidal activity. However, if the fish are immunized with the same bacterium after 4 weeks of exposure, the bactericidal activity at week 11 is maintained/restored to levels seen in control non-exposed dab. This implies that either immunization directly prevented the suppressive effect on the kidney leukocytes or that certain afferent immunological pathways were activated and able to antagonise the effects of the suppressive signals. Our studies on *in vitro* temperature effects (Hardie *et al.*, in press) have found that low temperatures inhibit macrophage respiratory burst activity (Figure 3), This requires at least 24 hr if cells are collected from fish at relatively warm temperatures (e.g. 14°C). Nevertheless, cells do respond to MAF supernatants at low temperatures (6°C) and show a relatively larger increase in activity compared with cells cultured at higher temperatures (Figure 3). The biological relevance of this finding is uncertain, however, since cells from fish kept at low temperatures (7°C) fail to produce MAF when cultured at equivalent (6°C) temperatures *in vitro* (Hardie *et al.*, in press). Thus it appears that potentially MAF is not generated at low temperatures although macrophages can respond to it once produced.

Stress is also known to inhibit fish phagocyte bactericidal pathways and microbicidal activity and our laboratory has recently shown this to be the case for Atlantic salmon head kidney phagocytes (Thompson *et al.*, 1993). In this study we examined the possibility that vitamin C might reduce the inhibitory effects of stress on the immune system, through a speculated brake on cortisol production. However, the degree of inhibition observed was not influenced by the level of dietary vitamin C.

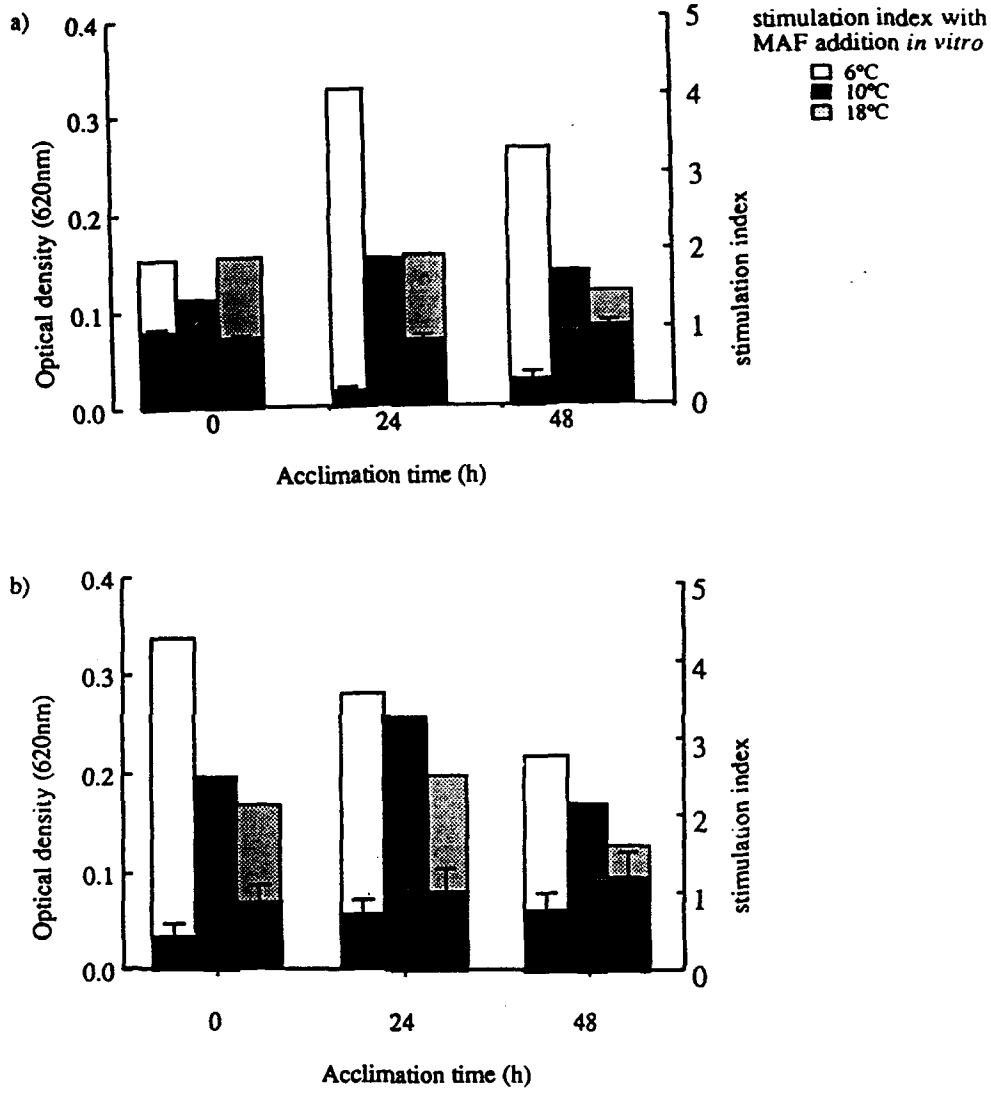

Figure 3. Respiratory burst activity of macrophages obtained from fish at **a)** 14°C or **b)** 7°C and cultured at varying *in vitro* temperatures for 0, 24 or 48 hr before addition of MAF-containing supernatants. Basal respiratory burst activity (solid bars/ OD 620 nm) was inhibited at cold temperatures using all culture regimes when cells were collected from 7°C-acclimated fish; ROS production was also depressed when the cells from fish kept at 14°C were acclimated to cold temperatures for 24 hr or more. MAF activity was expressed as a stimulation index, by dividing the respiratory burst activity of macrophages incubated with supernatants from antigen-stimulated leukocytes with that from macrophages incubated with control supernatants. Note that MAF induces an increase in respiratory burst activity under all regimes and that there is a relatively larger increase in activity from the cold temperature adapted cells. n=4 fish for each bar.

Unlike the above experiment with dab, cells from immunized fish were not tested for their bactericidal activity and so it is not known whether this may have overcome the suppressive effects. In addition, whether the production of macrophage activating cytokines is itself inhibited by stress has still to be examined.

Potential for modulating macrophage accessory function

Little is known about the modulation of antigen-presentation by fish macrophages. However, antigen-presentation does appear to be genetically restricted, since allogeneic macrophages cannot effectively perform this function (Vallejo *et al.*, 1992), and so it is likely to involve MHC class II products as in mammals. Recently, it has been possible to demonstrate that mRNA to the class IIβ chain is present in fish head kidney macrophages (personal communication Mr. F. Hepkema, University of Aberdeen). So it is now possible to investigate whether class II expression can be modulated in fish macrophages, and whether this has an impact on accessory cell function.

CONCLUSIONS

Whilst it should be apparent from this review that knowledge on the activation of fish macrophages has increased over the last few years, molecular biology has still to have a major effect in this area of fish immunology. It is likely that the identification of fish cytokine genes that are involved in macrophage activation and that are switched on in macrophages during activation will have an enormous impact over the next few years.

ACKNOWLEDGEMENTS

Many thanks to Celltech Ltd, for the generous gift of the anti-TNFα receptor monoclonal antibodies, and to Dr. J. Glamann (Statens Seruminstitut, Copenhagen) for sequence data and primers for the class II MHCβ chain gene of rainbow trout. Thanks also go to Dr. Ian Ashton and Dr. Andrew Rowley for Figure 1, Mr. Matthew Marsden for Figure 2 and Dr. Laura Hardie for Figure 3.

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Chapter 6

Macrophage-Colony Stimulating Activity in Rainbow Trout (*Oncorhynchus mykiss*) Serum

Hiroshi Kodama, Masafumi Mukamoto, Tsuyoshi Baba and Donny M. Mule[1]

Laboratory of Veterinary Immunology
Department of Veterinary Science, College of Agriculture
University of Osaka Prefecture, Sakai, Osaka 593, Japan

[1]Department of Paraclinical Studies, School of Veterinary Medicine
University of Zambia, Lusaka, Zambia

ABSTRACT

Hematopoietic colony-stimulating activity of rainbow trout (*Oncoryhnchus mykiss*) serum was examined using semi-solid agar culture. When normal head kidney cells were cultivated in the presence of trout serum obtained from fish injected with bacterial-derived lipopolysaccharide (LPS), colonies composed of mononuclear cells developed eight days after the cultivation. The colony-stimulating activity of the serum reached a peak one day after injection of LPS. The numbers of colonies then decreased gradually over time. Smaller numbers of colonies developed in culture with added normal trout serum, and a few colonies were observed in the absence of the serum. The number of colonies formed were dependent on serum concentration and the number of cells in culture. Morphological observation, phagocytic uptake and intracellular enzyme staining revealed that the cells in these colonies were of macrophage/monocyte lineage. Intraperitoneal injection of Freund's complete adjuvant or muramyl dipeptide to rainbow trout also resulted in the enhanced colony-stimulating activities of the serum, which reached a peak one and two days after the injection, respectively.

INTRODUCTION

Clonal proliferation of lymphocytes, macrophages and granulocytes, as well as other hematopoietic cells in semi-solid agar have been reported in mammals (Ihle *et al.*, 1982; Metcalf, 1971; Nicola *et al.*, 1983; Stanley *et al.*, 1977). This technique enabled the study of the mechanisms of cell differentiation, characterization of the cells, and determination of growth requirements of hematopioetic cells. Growth factors (colony-stimulating factors) have an effect on the differentiation of mammalian hematopoietic precursors and have been identified as a group of glycoproteins based on the types of cells that occur in the colonies; M-CSF (Stanley and Heard, 1977), G-CSF (Nicola *et al.*, 1983), GM-CSF and interleukin-3 (Ihle *et al.*, 1982). Further, CSFs are produced in the sera of animals after stimulation with various immunostimulators, followed by enhancment of

the function of immunocompetent cells (Metcalf, 1971, Yamaguchi *et al.*, 1988a, 1988b; Young *et al.*, 1990). However, very little is known about the ontogeny of fish hematopoietic cells, and humoral factors that induce the differentiation of the cells. Cultivation of cells in semi-solid agar has been reported in rainbow trout and carp lymphocytes (Caspi *et al.* 1982; Finenan and Mulcahy, 1987), and in carp head kidney cells (Moritomo *et al.*, 1992, 1993). In the present study, we analyzed the regulatory mechanisms of rainbow trout hematopoiesis by soluble factors in trout serum by cultivation of head kidney cells in semi-solid agar. Colony-stimulating activity of the serum was examined and the cell types of the individual colonies characterized. Further, the effect of injection into fish of lipopolysaccharide (LPS), Freund's complete adjuvant (FCA) and muramyl dipeptide (MDP) derivative on the colony-stimulating activity of trout serum, was examined.

MATERIALS AND METHODS

Fish and injection of immunostimulants

Rainbow trout, weighing about 100 g, were obtained from a commercial farm. The trout were acclimated to laboratory conditions for several weeks in 400 L well-aerated, plastic aquaria that were filled with dechlorinated tap water at a flow rate of 90 L /hr at a water temperature of 13°C.

Fish were injected intraperitoneally with MDP-Lys (L18) (N-acetylmuramyl-L-alanyl-D-isoglutaminyl-N-stearoyl-L-lysine), kindly provided by Dr. I. Azuma, Institute of Immunological Science, Hokkaido University, Sapporo, Japan, at a dose of 200 µg MDP/0.5 mL in phosphate buffered saline (PBS, 150 mM, pH 7.4) (Kodama *et al.* 1993). Another group of fish were injected with 200 µg/0.5 mL *Escherichia coli* (O111: B4) LPS (Difco Laboratories, Detroit, Mich.) or 0.5 mL FCA (Wako Pure Chemical Industries, Osaka, Japan). Control fish were injected with 0.5 mL of diluent.

Colony stimulating assay

Single cell suspensions of isolated head kidney cells were prepared in Eagle's minimum essential medium (MEM; pH 7.4, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 100 units of penicillin, 100 µg of streptomycin per mL of the medium and 1% fetal calf serum (FCS). The cell suspensions were carefully overlayed on a 1.060 - 1.070 discontinuous density Percoll solution (Pharmacia LKB Biotechnology, Uppsala, Sweden) in MEM. After centrifugation at 300 x g for 30 min, the interface fraction was collected and washed twice with Hank's balanced salt solution (HBSS; pH 7.4, Nissui Pharmaceutical Co.) containing 1% FCS. Cells were resuspended in α-MEM (pH 7.4, Gibco Laboratories, Grand Island, NY) containing 1% FCS.

The cell suspensions containing 2.5 x 10^5 cells/0.05 mL, 0.05 mL of trout serum and 0.1 mL of FCS were mixed with 0.3 mL of 1.67% methyl cellulose - α-MEM solution (4,000 cp, Wako Pure Chemical Industries) in 12-well tissue culture plates (Corning, NY). Control wells were prepared in the same manner exept for the addition of α-MEM instead of trout serum. The plates were incubated at 20°C for 8 days in an incubator supplemented with 5% CO_2 and 95% air.

Cell staining

Colonies formed in methyl cellulose were collected using a micropipette. Colonies suspended in MEM containing 50% FCS were cytocentrifuged (Cytospin, Shandon, Pittsburgh, Pa.), fixed with methanol and stained with Giemsa solution.

For peroxidase staining, colonies were fixed with acetone-formalin buffer (45% acetone, 25% formalin in 10 mM sodium-potassium buffer [Na_2HPO_4 + KH_2PO_4], pH 6.6). Colonies were then stained with 0.05% 3,3'-diaminobenzidine tetrahydrochloride solution in 200 mM Tris-HCl buffer (pH 7.6) for 10 min at room temperature. Hydrogen peroxide solution was added to the substrate solution at a final concentration of 0.1% immediately before staining. After washing with PBS for 5 min, the cells were counterstained with Giemsa solution.

Nitroblue tetrazolium (NBT) reduction was performed to evaluate the production of active oxygen species by colony-forming cells (Tokunaga *et al.*, 1992). Suspensions of colonies (0.1 mL, collected as described above) were mixed in test tubes with 0.1 mL of 0.1% NBT solution prepared in HEPES-saline-glucose buffer (17 mM HEPES, 120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, pH 7.4). The mixture was incubated at room temperature for 30 min, then fixed with 0.2 mL of 1% paraformaldehyde and 0.02% glutaraldehyde in PBS for 30 min. Suspensions were mounted on glass slides, covered with coverslips and evaluated for the presence of blue precipitates intracellularly.

Non-specific esterase staining was done as follows (Tokunaga *et al.*, 1992). A solution of 4% pararosanilin in 2 N HCl (80 µL) and 4% $NaNO_3$ in distilled water (80 µL) was mixed with 9.5 mL of 70 mM phosphate buffer, and the pH adjusted to 6.0 with NaOH. To this mixture, 0.5 mL of 2% α-naphthyl butyrate (dissolved in methyl cellosolve) was added, and then filtered (pore size 0.45 µm). Cytocentrifuged preparations of colonies on glass slides fixed with acetone-formalin buffer were stained with the staining solution described above for 45 min at room temperature. After washing the slides with running water, they were air dried and then observed microscopically for the presence of α-naphthyl butylester (α-naphthol cleaved from α-naphthyl butyrate by esterase, is coupled to pararosanilin and forms an insoluble colored dye).

Phagocytosis assay

To colonies suspended in MEM containing 10% FCS (50 to 100 colonies/0.5 mL) were added 5 µl of lumisphere (Toray Techno Co., Shiga, Japan), which contained 2 x 10^6 beads. Microspheres that had been opsonized with fresh rainbow trout serum at 20°C for 30 min were used for this study. The mixtures were incubated at 20°C for 2 hr, and the suspension of colony-forming cells mounted on a glass slide was observed for phagocytic activity.

RESULTS

Formation of colonies

Figure 1 shows the colony-forming cell response of trout head kidney cells cultivated for 8 days in the presence of rainbow trout serum. Small colonies were observed after 4 days. The size of colonies and the number of cells in the colonies increased and colony formation was dependent on the number

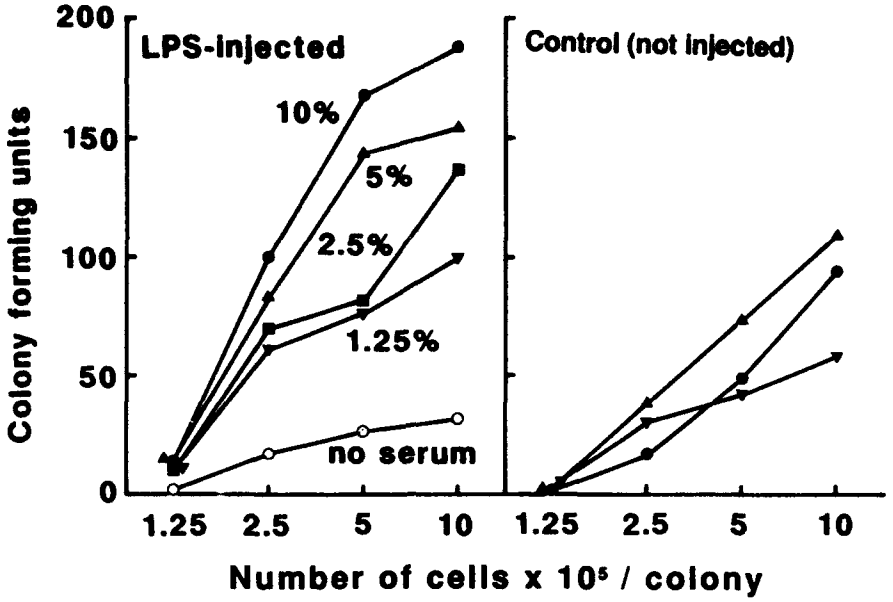

Figure 1. Colony-forming cell responses of head kidney cells in semi-solid agar culture. Cells were cultivated for eight days in the presence of different concentrations of LPS-stimulated rainbow trout serum or normal trout serum. Colony formation in the absence of the serum is also shown. The results are shown as mean numbers of colonies from four separate experiments.

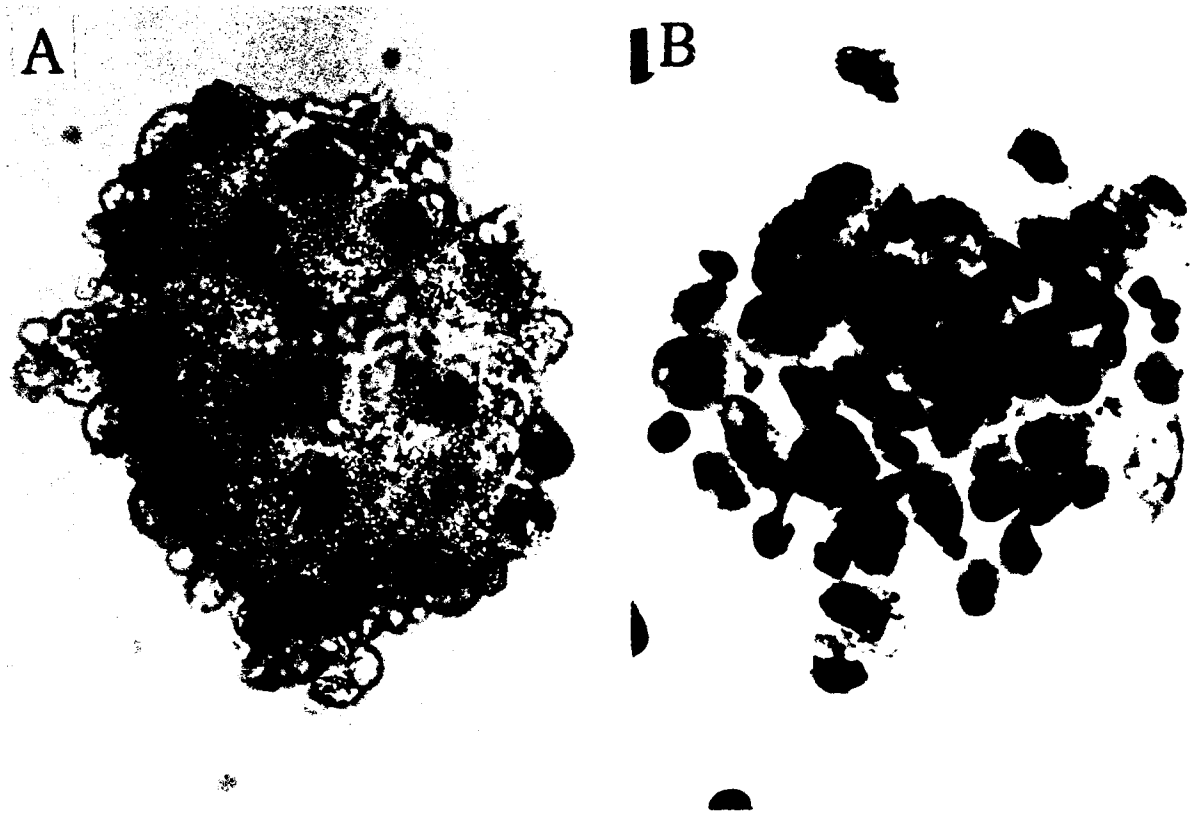

Figure 2. Phagocytic activity of colony-forming cells against opsonized lumisphere beads (A) (x 1,800). A colony of rainbow trout head kidney macrophages eight days after cultivation (B) (x 2,100).

of cells seeded and concentration of trout serum used. Serum from fish collected one day after LPS injection showed higher colony-stimulating activity than uninjected fish. Poor colony formation occurred when fish serum was not added.

Characteristics of colony-forming cells

Figure 2 shows the morphology of the colonies and the phagocytic activity of the cells within the colonies. Most of the cells phagocytosed numerous microspheres. They were observed very clearly in the cytoplasm (A). Giemsa staining revealed that the cells were a homologous cell type (B); round, possessing large oval or horseshore-like nuclei, and numerous cytoplasmic vacuoles. The cells reduced NBT (O_2- positive), and were negative or weakly positive for peroxidase and weakly positive for non-specific esterase (data not shown). These results suggest that the cells constructing the colonies are of the monocyte/macrophage lineage.

Effect of immunostimulants on colony formation

Fish sera obtained 1 to 8 days after intraperitoneal injection of either LPS, FCA, or MDP were examined at 10% concentration for their colony-stimulating activities. The largest number of colonies developed when the serum was collected from fish 1 day after IP injection of LPS or FCA. The number of colonies then decreased gradually over time (Figure 3). Serum from fish injected with MDP had the greatest colony-stimulating activity 2 days after injection. Table 1 shows types of colonies formed in culture. 98 to 100% of the colonies were macrophage/monocyte colonies whether formed in the presence of normal trout serum or with serum from fish injected with

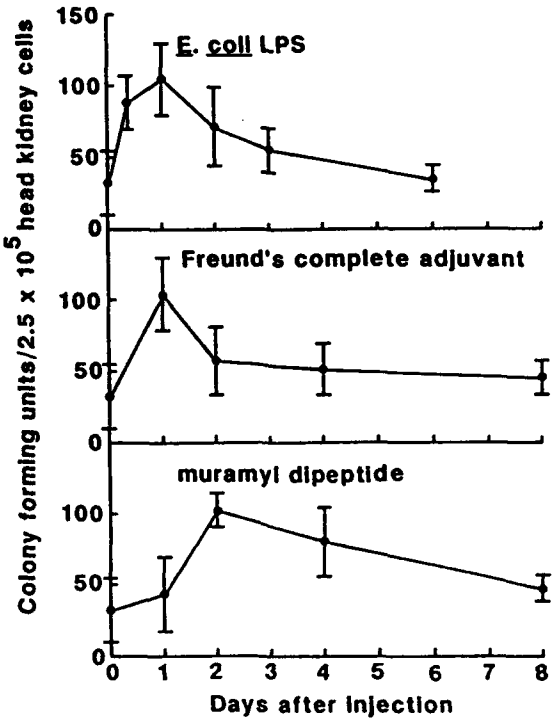

Figure 3. Time course study of colony stimulating activity in the serum of rainbow trout injected with LPS, FCA or MDP.

Table 1
Types of colonies formed in semi-solid agar cultures in the presence of rainbow trout serum

Rainbow trout serum	Number of colonies examined	Colony type (%)[1]		
		Macrophages	Macrophages/ Granulocytes	granulocytes
LPS-injected	113	111 (98%)	2 (2%)	0 (0%)
FCA-injected	8	8 (100%)	0 (0%)	0 (0%)
MDP-injected	23	23 (100%)	0 (0%)	0 (0%)
normal	112	110 (98%)	1 (1%)	1 (1%)
no serum	13	13 (100%)	0 (0%)	0 (0%)

[1]Suspensions of colonies were added lumisphere beads and incubated at 20°C for 30 min before staining with Giemsa solution. Cell types composing each colony was determined by phagocytic activity and morphological features of the cells.

immunostimulants, or with no serum. A few colonies were granulocyte colonies or a mixture of macrophages/monocytes and granulocytes.

DISCUSSION

The present experiments studied the mechanisms which regulate differentiation and multiplication of hematopoietic cells in rainbow trout head kidney cells. We used a semi-solid agar culture technique since it is advantageous for the analysis of the process of hematopoiesis and ontogeny of immunocompetent cells *in vitro* (Ihle *et al.*, 1982; Metcalf, 1971; Nicola *et al.*, 1983; Stanley *et al.*, 1977; Metcalf, 1971, Yamaguchi *et al.*, 1988a, 1988b).

Rainbow trout serum had an effect on the formation of head kidney cell colonies. Cells composing colonies were identified as macrophages or monocytes by morphology, tests positive for phagocytosis, O_2- production and non-specific esterase staining. Further, actin, cytoskeletal protein which participate in cell movement, was clearly observed in cell membrane and cytoplasm as ruffles and, in some cells, microspikes when the cells were stained with fluorescent-labeled phalloidin (data not shown). Recently, Moritomo *et al.* (1992, 1993) reported hematopoietic colony-forming activity in carp serum; head kidney cell colonies contained mature and immature granulocytes. Though the differences of cell types observed in their experiment and present study is not clear, humoral factors in fish serum might be involved in differentiation of various types of hematopoietic cells in these fish species.

Requirement of growth factor in the clonal culture of fish lymphoid cells has been suggested (Caspi *et al.*, 1982; Finegan and Mulcahy, 1987); *e.g.* peripheral blood lymphocytes were successively cultivated in soft agar in the presence of mitcgens (phytohemagglutinin or LPS) which stimulate the cells to produce growth factors. Since few hematopoietic cell colonies developed in the culture without rainbow trout serum (present results) or carp serum (Moritomo *et al.*, 1992, 1993), it seems that fish serum contained growth factor(s) for hematopoietic cells. Colony-forming activity in trout serum in the present study might be attributed to M-CSF-like factor.

We investigated the effect of immunostimulators (LPS, FCA and MDP) on the formation of hematopoietic cell colonies. Since these agents are known to activate lymphocytes and macrophages to produce CSFs in mammals (Metcalf, 1971, Yamaguchi *et al.*, 1988a), colony-stimulating activity by stimulated fish serum was examined. The activity of the serum increased when fish were injected with either of these immunostimulators, indicating that a CSF-like factor was produced in fish. The number of colonies developed, reached a peak one day (LPS and FCS) or two days (MDP) after the administration. The effects of the CSF-like factors on the activation of immunocompetent cells in fish is of interest both from the point of view of phylogeny and also of fish health. In murine, MDP increased the level of serum CSF, stimulated the proliferation of stem cells in bone marrow, and granulocytes and monocytes in peripheral blood (Yamaguchi *et al.*, 1988a). In addition, MDP stimulated murine macrophages to induce CSF in the presence of T cells (Yamaguchi *et al.*, 1988b). Recently, Moritomo *et al.* (1992) reported that injection of FCA into carp caused a high level of serum colony-stimulating activity which produced granulocyte colonies in semi-solid agar. Purification and characterization of these CSF-like growth factors, and clarification of the regulatory mechanisms of cell differentiation by these factors should be performed.

The mechanism of antigen processing by macrophages, macrophage activation and host resistance to microbial infection are the subjects of utmost concern in fish immunology (Chung and Secombes, 1987, Graham and Secombes, 1988; Vallejo *et al.*, 1992). We reported the enhancing activity of MDP on the macrophage functions in rainbow trout (Hirota *et al.*, 1993; Kodama *et al.*, 1993). Head kidney macrophages from fish injected with MDP showed significant chemotactic and phagocytic activities, and enhanced O_2- production. Injection of MDP into fish provided protection against challenge with the fish pathogen *Aeromonas salmonicida*. These results, together with the present findings, suggest that fish injected with MDP, FCA or LPS produce soluble factors such as CSFs, or macrophage or lymphocyte-activating factor which might induce differentiation of hematopoietic progenitors and activate immunocompetent cells which enable fish to acquire resistance against microbial infection through the activation of these cells.

ACKNOWLEDGEMENT

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Chapter 7

Immune Response Enhancement in Channel Catfish, *Ictalurus punctatus*, using β-glucan From *Schizophyllum commune*

A. Jerald Ainsworth, Cheng Ping Mao, Carolyn R. Boyle

College of Veterinary Medicine, Mississippi State University
Mississippi State, MS 39762-9825 USA

ABSTRACT

β-Glucans have been shown to have immunopotentiating effects in a variety of animal species. The ability of β-glucan derived from *Schizophyllum commune* to enhance immune responses of channel catfish was determined by quantitating antibody titer to *Edwardsiella ictaluri*, reduction of nitroblue tetrazolium (NBT), serum lysozyme concentrations, and mortality in challenge trials. Intraperitoneal (IP) injection of β-glucan resulted in an initial accumulation of neutrophils in the peritoneal exudate that declined over a four day period. In addition, macrophage numbers increased over the four day period and were more abundant in the exudate on day 4 post-injection. Fish fed rations supplemented with 0.1% β-glucan had significantly higher antibody titers to *E. ictaluri* than fish fed control or 1.0% β-glucan supplemented rations. This response was found to be related to the weight of the fish. The capacity of β-glucan to induce the production of intracellular superoxide was determined by reduction of NBT. β-Glucan *in vitro* caused the reduction of twice as much NBT per 10^6 neutrophils compared to *E. ictaluri*; phorbol myristate acetate caused a four-fold increase in NBT reduction compared to β-glucan. Serum lysozyme concentrations were not significantly affected by β-glucan-supplemented rations, however there was a trend for fish receiving β-glucan to have higher lysozyme concentations than control fish. In immersion challenge trials using *E. ictaluri*, β-glucan-supplemented rations did not significantly alter mortality of fish weighing 18 ± 2 g when compared to catfish fed control rations. β-Glucan may be useful when used in conjunction with vaccines.

INTRODUCTION

The major factor contributing to economic loss in fish culture is mortality from disease, infectious and non-infectious. Even successful fish farmers must contend with a significant loss from disease. In the Mississippi catfish industry, losses from both infectious and non-infectious diseases amount to $40 million annually. Much of this loss can be attributed to infectious diseases such as enteric septicemia of catfish (Hawke, *et al.*, 1981), *Flexibacter columnaris*, motile *Aeromonas* septicemia and channel catfish virus disease. Despite the significant economic losses within the

catfish industry due to infectious diseases, fish health managers are limited in their approaches to preventing and treating diseases because of a lack of therapeutic modalities. Only two antibiotics are presently approved for use in catfish culture and the danger of increasing resistance to antibiotics occurs. Likewise, vaccine development and application has been slow and minimally effective. Several immunomodulators, such as modified complete Freund's adjuvant (Olivier *et al.*, 1985), FK-565, a lactoyl tetrapeptide (Kitao and Yoshida., 1986), Ete, an extract from the tunicate *Ecteinascidia turbinata* (Davis and Hayaska., 1984), and glucan (Robertsen *et al.*, 1990; Yano *et al.*, 1989), have proved effective in some fish species by enhancing phagocytic responses. However, it is not known if biological response modifiers can enhance or potentiate the cell-mediated or humoral immune responses in channel catfish (Badwey *et al.*, 1986; Carelli *et al.*, 1981; Fridkin *et al.*, 1977). The feeding of an immunomodulator alone or in conjunction with a vaccine could induce an enhanced immune response thus providing for better protection from disease.

Glucan, a polysaccharide polymer of glucose derived from various organisms are known to have neutrophil and macrophage activating capability (Czop *et al.*, 1985a; Czop *et al.*, 1985b). Depending on the extraction method, glucan has varying ratios of α- or β-1,6 glycosidic side chains. Activation of mammalian phagocytes by glucan occurs through the binding of a specific glucan receptor on the cell surface (Williams *et al.*, 1986). Channel catfish have a similar β-glucan binding receptor (Ainsworth, In press). In mammals, binding initiates cell activation and the alternate complement pathway. Binding of multivalent β-1,3 glucan to its receptor enhances phagocytosis by macrophages and neutrophils in at least two ways: 1) activation of complement via the alternative pathway (Czop *et al.*, 1985a) and 2) stimulation of the metabolism of endogenous arachidonic acid through the 5-lipoxygenase pathway (Williams *et al.*, 1984, 1985). Complement components C3b and C3bi generated during complement activation are opsonins enhancing phagocytosis while leukotriene B_4, a metabolite of arachidonic acid, stimulates *in vitro* adhesion (Dahlen *et al.*, 1981; Goetzel *et al.*, 1983), migration (Dahinden *et al.*, 1984; Ford-Hutchinson *et al.*, 1980), aggregation (Ford-Hutchinson, *et al.*, 1980) and granular enzyme release (Naccache *et al.*, 1989; Showell *et al.*, 1982) by neutrophils. Di Luzio (1985) cites in a review article numerous examples of glucan significantly increasing survival in experimentally infected animals. In summary, the ability of laboratory animals to cope with malignancy and immunosuppression has been beneficially enhanced with particulate and high-molecular-weight glucan (Di Luzio *et al.*, 1984, 1985).

The research mission of our laboratory is to provide the catfish industry with cost-effective measures to predict, prevent, and decrease the susceptibility of catfish populations to disease. The purpose of this study was to determine the capacity of a commercially available β-glucan product derived from *Schizophyllum commune*, VitaStim-Taito® (Taito Co. Ltd., Tokyo, Japan), to function as an immunostimulant in channel catfish.

MATERIALS AND METHODS

Experimental animals

Channel catfish (*Ictalurus punctatus*, Rafinesque) of various weights were used in the experiments described below. The specific weight of catfish used in each experiment has been noted. Catfish were acclimated for 7 days in flow-through tanks maintained at 26 ± 1°C before initiating any experiments. Acclimating fish were fed commercial catfish ration (Delta Western Feed Mill, Indianola, MS) at 5% of body weight on a daily basis. Care and use of animals also followed the

guidelines established for biomedical research by the National Institutes of Health-Public Health Service.

In vivo stimulation and collection of peritoneal exudate cells

Fifteen catfish weighing 180 ± 20 g were maintained as noted above. Prior to intraperitoneal injection of VitaStim-Taito (VST®, Taito Co., Toyko, Japan) fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO). VitaStim-Taito® was suspended in sterile calcium-magnesium-free Hanks' balanced salt solution, pH 7.2, mOsm 275, (GIBCO, Grand Island, NY) without phenol red indicator (HBSS-CMF) at a concentration of 125 µg/mL. Each fish received one IP injection of 250 µg of VST® at time zero. Three fish were sampled at 24, 48, 72, and 96 hr after the IP injection of VST®. For collection of peritoneal exudate cells, fish were anesthetized with MS-222 before peritoneal lavage. The fish were bled via the caudal vein to reduce red blood cell contamination of the peritoneal exudate. An abdominal incision was made on the ventral surface and the peritoneal cavity was rinsed with 40 mL of cold sterile HBSS-CMF. The lavage fluid was centrifuged at 400 x g for 20 min at 22°C and the cell pellet resuspended in 2 mL of HBSS-CMF. Cells were counted using a hemocytometer and viability determined by 0.2% trypan blue exclusion. The final cell preparations were diluted in HBSS-CMF to a concentration of 5 X 10^6 cells/mL to be used for slide preparations.

Cytocentrifuge slide preparation

Cytocentrifuge slide preparations were prepared using a cytocentrifuge (Shandon Cytospin II, Sewickley, PA, USA) in which the casettes were prewashed with 0.3 mL of HBSS-CMF at 200 xg (1500 rpm) for 3 min. To prepare slides 0.1 mL of cell suspension, 0.05 mL of fetal calf serum, and 0.2 mL of HBSS-CMF were added to the casettes and centrifuged for 2 min at 22 xg (500 rpm). Slide preparations were air dried at 22°C and stained with Wright's, Sudan black B, and non-specific esterase stains. Following staining, differential counts for macrophage and neutrophil identification were done by viewing a total of 300 cells per slide (two slides per preparation). Differentiation of leukocytes was based on morphological and differential staining characteristics.

Preparation of catfish rations

Preparation of VST® supplemented feed was accomplished by first mixing VST® with an unmedicated premix (Abbott Laboratory, North Chicago, IL). The VST®-unmedicated premix was combined with a 50:50 mixture of trout chow (Purina Mills, Inc., St. Louis, MO) and catfish fingerling crumbles (MFC Services, Madison, MS). The final concentration of VST® in this feed was 0.1% or 1% (w/w). The rations were thoroughly mixed dry and water was added for final mixing. The mixture was extruded through a one-eighth inch grate affixed to a food chopper (Hobart, #4612) and the pelleted feed allowed to air dry 24 hr before bagging and storing at -20°C until needed. Control rations were prepared in a similar manner except VST® was not included.

Absorption of VST by the gastrointestinal (GI) tract

Upon acclimation 20 channel catfish weighing 40 ± 5 g were divided into groups of 10 fish per group. One group continued to receive commercial catfish rations while the experimental group received VST® (0.1% w/w) supplemented rations. Ten fish from each feed group had portions of the pyloric, middle and rectal intestines removed 1 and 4 days after starting VST® feeding.

Hematoxylin and eosin (H & E), Wrights, Periodic acid Schiff (PAS), Sudan black B, and an immunohistochemical staining method were done on each tissue. Tissues for H & E and PAS examinations were fixed in phosphate-buffered formalin (Culling, 1974) and processed by the Histopathology Laboratory, College of Veterinary Medicine, Mississippi State University. Specimens for Wright's, Sudan black B and immunohistochemical stains were embedded in OCT® compound (Miles Inc., Elkhert, IN), snap frozen in liquid nitrogen, and stored at -70°C until sectioned. Five µm cryostat sections were made by using a freezing ultramicrotome. The tissue sections were air dried, heated to 50°C, rinsed once in 0.01M phosphate buffered saline, pH 7.4, mOsm 280, (CF-PBS) and processed according to the particular staining method.

Wrights and Sudan black B stains were processed as previously described (Ellsaesser and Clem, 1986). Immunohistochemical staining was done as described below with minor modifications. Briefly, air dried cryostat sections were incubated in diluted fluorescein isothiocyanate conjugated concanavalin A (1:10 , FITC-Con A, Sigma chemical Co., St. Louis, MO) at 22°C for 60 min in a dark humid chamber. The sections were washed in CF-PBS for three 10 sec immersions. Anti-catfish neutrophil monoclonal antibody 51A (Ainsworth *et al.*, 1990) diluted 1:10 in CF-PBS containing 3% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) was applied to the sections for 1 hr incubation at 22°C in a dark humid chamber. The sections were washed again in CF-PBS for three 10 sec immersions. Finally, the tissue sections were reacted at 22°C for 1 hr with goat-anti mouse polyvalent immunoglobulin rhodamine conjugate (GAM Ig-R, Southern Biotechnology Associates Inc., Birmingham, AL) diluted in CF-PBS containing 3 % BSA. Slides were washed 3 times with CF-PBS, drained, and immediately coverslipped and observed using an epi-fluorescent microscope.

Bacterial preparations

Mortality trials (described below) were done using a virulent clinical isolate of *Edwardsiella ictaluri* that was plated on tryptic soy agar containing 5% sheep blood (Difco Laboratory, Detroit, MI). After growth on the plate, a single isolated colony was inoculated into brain heart infusion (BHI) broth (Difco Laboratory, Detroit, MI) and grown 18 hr at 27 ± 1°C. A portion of the 18 hr culture was added to 600 mL of BHI broth for expansion and used in mortality trials. Once the optical density (O. D.) of the culture at 540nm exceeded 1.2, the culture was diluted to an optical density of 1.0 for use in immersion mortality trials. The actual bacterial density was determined by a plate count method.

Live and heat-inactivated bacterins of *E. ictaluri* (ATCC 33202, American Type Culture Collection, Rockville, MD) were prepared by culturing an isolated colony in BHI broth at 27 ± 1°C. Exponential phase cultures were used at an O.D. 540nm that would yield approximately 10^6 to 10^7 bacteria/mL of tank water (vaccination procedure described below). The bacterin was produced by heat-inactivating (killing) a culture for 3 hr at 60°C. Indirect fluorescent antibody (IFA) testing (Ainsworth *et al.*, 1986) and traditional bacteriological identification were done to confirm the purity of all cultures.

Intracellular superoxide quantitation

A modification of the procedure described by Secombes (1990) was used to quantitate the production of intracellular O_2- using nitroblue tetrazolium (NBT) in kidney neutrophils. Neutrophils were isolated from normal fish fed the control diet. Anterior kidney preparations were layered

onto 1.060 g/mL and 1.080 g/mL discontinuous gradients and centrifuged at 400 xg for 25 min at 22°C. The neutrophils were removed from the 1.066-1.080 g/mL interface, washed and counted. Assays were run in wells of microtiter plates as follows with reagents added in the order listed: 50 µl bovine calf serum, 100 µL HBSS with 2mM CaCl, 100 µL NBT, and 100 µL of isolated neutrophils. In cases where stimulants (inactivated *E. ictaluri*, phorbol myristate acetate, and VST®) were included a 50 µL quantity of each was used and an appropriate quantity of HBSS deleted from the assay. Phorbol myristate acetate (PMA) was dissolved in DMSO and used in the assay at a final concentration of 50 ng/well. VST and *E. ictaluri* were suspended in HBSS and used at a final concentration of 20 µg/well and a 20:1 ratio to neutrophils or 2 x 10^7 organisms/well respectively. Assays were incubated at 27°C, 5% CO_2, 90% humidity for 60 min. After incubating, the plates were centrifuged for 3 min at 600 xg to pellet cells and the supernate removed. To the pelleted cells 120 µL of KOH and 140 µL DMSO were added, vigorously mixed, and the plates centrifuged as before. Two hundred twenty-five µl of the supernate were transferred to a clean well and the absorbance determined using a spectrophotometric plate reader (Dynatech Instruments, Torrence CA) equipped with a 630nm filter. A standard concentration curve of known quantites of NBT dissolved in KOH and DMSO was run with each assay and read using a spectrophotometric plate reader equipped with a 630 nm filter. The optical density of each sample was converted to nM of NBT reduced and adjusted accordingly to reflect nM of NBT reduced per 10^6 neutrophils.

Determination of serum lysozyme concentration

Serum lysozyme was determined using the method of Yousif *et al.* (1991). Briefly, 15 µL serum samples from fish (the same samples used to determine antibody titers) were dispensed into wells in agarose containing *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO). The petri dishes were incubated for 20 hr at 22°C and zones of lysis measured. For calculation of lysozyme concentration in unknowns, standard zones produced by hen egg-white lysozyme were run and used to produce a standard curve.

Mortality trials using VST® supplemented rations

Two hundred and sixty channel catfish fingerlings weighing 18 ± 2 g were randomly assigned to 13, 150 L flow through tanks (20 fish per tank). Six of the tanks of fish received 0.1% VST® supplemented rations and 7 of the tanks received control rations. One of the 7 tanks was a sentinel tank and served to monitor techniques. Catfish were fed their assigned rations for 14 days before infecting with virulent *E. ictaluri*. On day 14 the catfish were fed 2% of body weight. On day 15 of the trial, the water volume of each tank was reduced to 25 L, the water turned off, and 200 mL of the prepared live *E. ictaluri* culture dispensed into each tank. Fish in each tank were immediately fed the appropriate rations. The tanks were held static for 2 hr and then refilled at a flow rate of approximately 1 L/min. The tanks were maintained as a flow-through system throughout the remainder of the trial. Fish were observed for an additional 14 day and mortalities recorded per day per tank. The cause of death was confirmed by bacteriological culturing.

VST® supplementation and antibody titer trials

Channel catfish were randomly assigned to 150 L flow-through tanks maintained at 26 ± 1°C. Twenty fish were assigned to each tank with 9 tanks of fish weighing 18 ± 2 g in Trial 1 and 12 tanks of fish weighing from 17.6 to 70.6 g in Trial 2. Fish were fed at the rate of 5% body weight

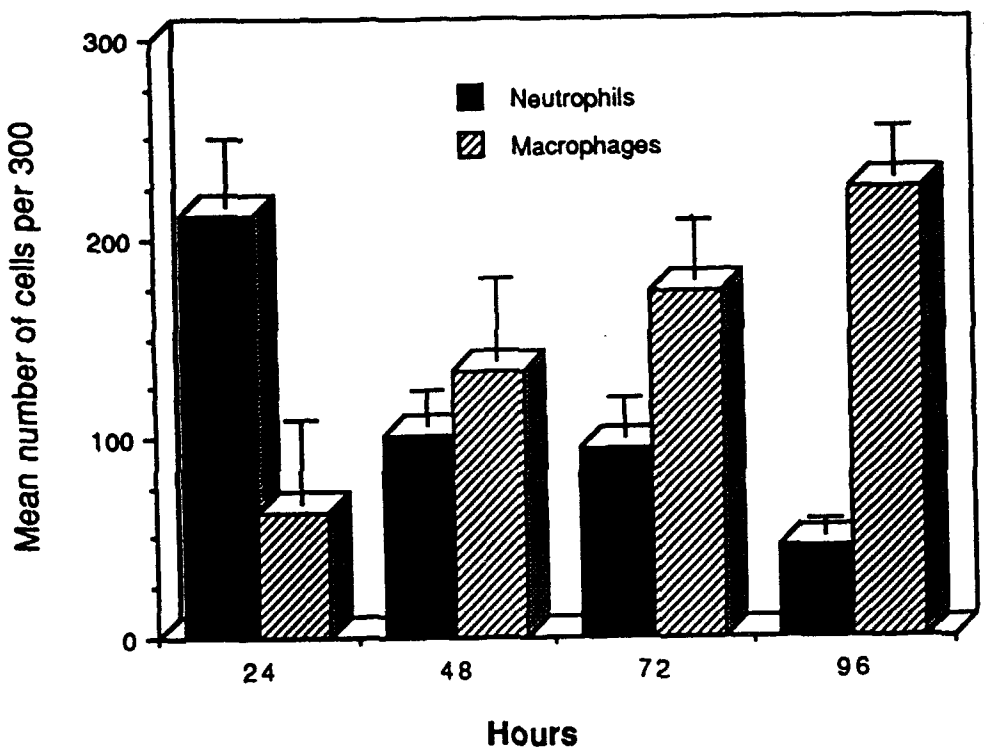

Figure 1 Injection of VST® into the peritoneal cavity of channel catfish caused the accumulation of neutrophils and macrophages. The majority of the cells in the peritoneal exudate fluid at 24 hr were neutrophils. Macrophages were the majority of cells in the peritoneal exudate at 48 hr and thereafter. Error bars represent the standard error of the mean, n=4.

with 0.1% VST® or 1.0% VST® supplemented or control rations for 6 days before vaccination. On day 7 the fish were fed 2% of body weight. On day 8 of the trial, the water volume of all tanks was reduced to 25 L and 200 mL of either live or killed bacterin added to the appropriate tanks. Each tank was immediately fed the appropriate rations at 5% body weight. The live bacterin was administered at a concentration of 5.5×10^5 organisms/mL of tank water. An identical volume of PBS was added to nonvaccinated tanks. All tanks were maintain static for 2 hr and the tanks returned to a flow through system at the end of the period. Catfish were bled 14 day after vaccination and the serum retained for determining serum antibody titer to *E. ictaluri*. Serum samples were assayed for antibody titer using a previously described enzyme-linked immunosorbent assay (ELISA) (Waterstrat *et al.*, 1989).

Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS Institute Inc., Cary, NC) software; all analyses were performed at the 10% level of significance. The effect of VST® administration on the accumulation of peritoneal exudate cells was analyzed using analysis of variance (ANOVA) for a completely randomized design and means were separated by the least significant difference (LSD) test. Lysozyme data analyzed using ANOVA for a completely randomized design were compared using the LSD. Mortality data were analyzed using a chi-square test of independence. Treatments were compared using percent mortalities in the first 3 day and over all day that deaths occurred in the mortality trials by constructing 90% confidence intervals for the difference between each pair of percentages. The effects of oral feeding of VST® on *E. ictaluri* log antibody titers were analyzed with ANOVA for a completely randomized design, with ELISA titers determined using a previously reported method (Snyder *et al.*, 1982; Waterstrat *et al.*, 1989). The means of log titers were compared using the LSD and were back transformed to obtain the geometric mean titers. A 90% confidence interval was also constructed for each geometric mean titer.

RESULTS

Characterization of the peritoneal exudate cells and GI tract tissues

The peritoneal exudate cells from VST® injected fish were collected at 24, 48, 72, and 96 hr and characterized morphologically based on Sudan black B and non-specific esterase staining. Neutrophils were the major cell type (70.6%) and significantly ($p<0.05$) higher than macrophages at 24 hr after IP injection (Figure 1); however, the numbers of macrophages were significantly higher than neutrophils at 72 and 96 hr. Ten to 20 % of the cells observed on each slide were undifferentiated, excluding the red blood cells. Intestinal sections stained by H & E, Wrights, and Sudan black B stains demonstrated no detectable difference in the distribution of intraepithelial leukocytes of control or VST® fed fish.

Reduction of NBT when using VST® as the stimulatant

The production of intracellular superoxide was determined by *in vitro* reduction of NBT by neutrophils upon exposure to PMA, *E. ictaluri*, or VST® .VST® β-glucan caused the reduction of 6.2 nM of NBT per 10^6 neutrophils. The non- stimulated control, positive control (phorbol myristate acetate), and *E. ictaluri* caused a reduction of 4.8, 24.3, and 4.0 nM per 10^6 neutrophils, respectively.

Serum lysozyme concentrations

A significant difference in lysozyme concentrations was noted between fish groups; however, the difference could not be attributed to supplementation of rations with VST®. The significant difference was related to the fish being exposed to the live bacterin vaccination. The mean ± SD lysozyme concentration for 80 fish that were not vaccinated was 278.6 ± 17.7, 250.3 ±15.9, and 271.0 ± 28.2 µg/mL for control, 0.1% VST® supplementation, and 1.0% VST® supplementation. Fish that were vaccinated with the live bacterin had concentrations of 1111.8 ± 344.1, 1244.5 ± 270.8, and 1430.1 ± 373.0 µg/mL for control, 0.1% VST® supplementation, and 1.0% VST® supplementation. The VST® supplementation appeared to cause an increase in serum lysozyme; however, as stated before the increase was not significant.

Mortality trials

In general, channel catfish fed VST® supplemented rations and challenged by immersion with live *E. ictaluri* did not have significantly lower percent mortalities ($p < 0.1$) when compared to catfish fed control rations (Table 1). Although no statistical differences in percent mortalities were demonstrated when comparing feed rations at the completion of the mortality trials, there was clear evidence in the trial that the control fed fish had a much higher mortality rate during the first two to 3 days when deaths began to occur.

Table 1
Effect of VST® administration on channel catfish mortalities following *E. ictaluri* challenge[1].

	8 days after challenge		18 day after challenge	
Rations	No. dead	Percent mortality[2]	No. dead	Percent mortality
1 % VST®	21	20.3a	56	54.4a
Control	35	37.6b	54	58.1a

[1]Fish weighing 18±2 g were challenged with 9.28 X 10^6 bacteria/mL of water.

[2]Values in percent mortality columns for this trial having different alphabetical designations are significantly different by LSD (10% level).

The effect of VST® feeding on *E. ictaluri* antibody titers

Edwardsiella ictaluri antibody titers of fish vaccinated with heat-inactivated bacterin were not significantly different ($p<0.05$) from the titers of non-vaccinated control fish regardless of the type of rations the fish received (Table 2). However, vaccination of catfish (regardless of rations received) by immersion in live *E. ictaluri* bacterin caused an increase in antibody titer when compare to catfish vaccinated with heat killed bacterin (Table 2). Live bacterins were used in the remainder of the study due to their ability to caused potent antibody responses. The type of rations a group of catfish received was also a factor in determining the antibody titer. Catfish receiving a diet supplemented with 1.0% VST® had antibody titers that were either significantly lower than fish in

Table 2.
Effects of live or heat-inactivated *E. ictaluri* vaccination and VST® supplementation on *E. ictaluri* antibody titers (Trial 1)

Rations[1]	Treatment[2]	Titer[3]	Lower 90%Confidence Limit	Upper 90% Confidence Limit
Control	not vaccinated	4.89c	2.35	10.19
1.0% VST	not vaccinated	0.85d	0.41	1.77
0.1% VST	not vaccinated	1.62d	0.73	3.59
Control	killed bacterin	1.24d	0.60	2.58
1.0% VST	killed bacterin	1.03d	0.49	2.19
0.1% VST	killed bacterin	1.15d	0.49	2.68
Control	live bacteria	1421.04a	670.01	3013.92
1.0% VST	live bacteria	462.97b	218.29	981.92
0.1% VST	live bacteria	2220.56a	1046.98	4709.64

[1]Fish were fed commercial catfish rations (control) or rations supplemented with 0.1 or 1.0% VST®.

[2]Fish were vaccinated according to the previously described protocol (Materials and Methods) with live or heat-inactivated *E. ictaluri*.

[3]Within analysis, titers having different alphabetical designations are significantly different by LSD (10% level) on log titer.

the control and 0.1% VST® supplemented groups or no different from the control group (Table 3). If the catfish were subdivided into groups based on weight (<35 g and >35 g) and significance in antibody titers determined based on the ration a group received, the results were more striking. There was no significant difference in antibody titer among diets (1% or 0.1% VST® supplemented, or control rations) of catfish weighing <35g. However, when considering catfish weighing >35 g, the group of catfish receiving 0.1% VST® supplemented rations had significantly higher antibody titers when compared to the other two ration groups. Regardless of weight the 0.1% VST® supplemented group had a significantly higher antibody titer when compared to the control ration group but not the 1.0% VST® supplemented group.

Table 3
Effects of vaccination with live *E. ictaluri* and VST®supplementation on *E. ictaluri* antibody titer (Trial 2)

Rations[1]	Vaccinated[2]	Statistical Analysis based on[3]	Titer[4]	Lower 90% Confidence Limit	Upper 90% Confidence Limit
Control	no	Fish >35 g	78.30c	46.19	132.73
Control	yes		3915.42b	2887.07	5310.05
1.0% VST	yes		3652.88b	2616.29	5100.18
0.1% VST	yes		6249.86a	4759.04	8207.70
Control	no	Fish <35 g	78.30b	34.37	178.39
Control	yes		5785.12a	3032.40	11036.67
1.0% VST	yes		6635.68a	3942.02	11169.99
0.1% VST	yes		398.57a	4656.91	18968.18
Control	no	All fish	78.30c	40.36	151.89
Control	yes		4491.01b	3300.15	6111.59
1.0% VST	yes		4923.35ab	3660.72	6621.48
0.1% VST	yes		6972.84a	5203.49	9343.82

[1]Fish were fed commercial catfish rations (control) or rations supplemented with 0.1 or 1.0% VST®.

[2]Fish were vaccinated according to the previously described protocol (Materials and Methods) with live *E. ictaluri*.

[3]Fish weighing 35 g had an average weight of 47.05± 9.16 g and fish weighing<35 g had an average weight of 30.02 ± 3.82 g.

[4]Within analysis, titers having different alphabetical designations are significantly different by LSD (10% level) on log titer.

DISCUSSION

VitaStim-Taito® used in the present study is a highly purified, partially depolymerized product derived from the mycelium of the yeast *Schizophyllum commune*. The important chemical constituent of VST® is β-1,3 glucan having β-1,6 glycosidic side chains. β-1,3 glucan from a variety of sources has been reported to enhance the resistance of carp and salmon to various bacterial infections (Nikl *et al.*, 1991; Robertsen *et al.*, 1990; Yano *et al.*, 1989) and for this reason was deemed important to investigate using channel catfish. It is believed that β-1,3 glucans act to enhance disease resistance by stimulating the macrophage component (Nikl *et al.*, 1991; Robertsen *et al.*, 1990), elevating the phagocytic activity of pronephric cells, and activating the alternative complement pathway (Yano *et al.*, 1989) in the fish immune system. However, the stimulating effect may differ with the composition of glucan, route of administration, dosage, timing of feeding, fish species, fish age, combination with bacterin, challenge method, bacterial concentration, etc.

The profile of cells observed at 24, 48, 72, and 96 hr in the peritoneal exudate of channel catfish administered an intraperitoneal injection of VST® mimicked the inflammatory process seen with other irritants and stimulants administered in a similar fashion. The cells initially found in the acute phase of inflammation are neutrophils with later stages resulting in the accumulation of macrophages

(Robbins, 1974). The ability of VST® to elicit neutrophils and macrophages indicates that it has irritant/stimulant properties.

Although the pathogenesis of many bacterial diseases of channel catfish is poorly understood, scientists agree that one of the major portals of entry into the fish is oral. If catfish ingest rations containing a substance, *e.g.* VST®, a reservoir of activated neutrophils and macrophages in the gut wall could prevent bacterial entry into the host; the bacteria being overwhelmed by the shear number of phagocytes. A response of this nature could be beneficial and result in a decrease in fish mortality. Based on the results of the peritoneal exudate study and the fact that some aquatic pathogens enter the host through the GI tract, an attempt was made to determine if VST® supplemented rations would elicit a similar cellular response in the gut wall of a channel catfish. Accumulations of neutrophils and macrophages in the pyloric, middle, and rectal portion of the intestinal tract of catfish after 1 to 4 day of VST® supplemented rations were not microscopically evident. Although a variety of stains were used, successful demonstration of increased phagocyte numbers was not evident. The lack of increased phagocyte numbers however, does not negate the potential positive benefits of VST®. There are several explanations for these results. Approximately 60% of the VST® is insoluble β-glucan that could potentially be sequestered in the gut wall. The insoluble VST® is a small particle having almost translucent properties making it difficult, if not impossible, to locate in tissue sections. Although special stains (ConA-FITC) were used in an effort to visualize the particles, the methods were unsuccessful. The kinetics resulting in a microscopically visible accumulation of neutrophils or macrophages in the gut wall are unknown relative to β-glucan. In our experiments gut tissues were removed from fish 1 and 4 day after feeding VST® supplemented rations which may have been too soon after administration. Continued feeding of the supplemented rations with later sampling may have given better results. Another possibility is that the β-glucan is not sequestered in the gut wall but is transported immediately to the spleen, liver, etc., or that it does not enter the gut wall and exits in the feces.

Two important facets of this research focused on the ability of β-glucan to reduce mortality in *E. ictaluri* challenged fish and to enhance antibody responses in *E. ictaluri* vaccinated fish. Chen and Ainsworth (1992) have previously shown that IP administration of β-glucan derived from *Saccharomyces cerevisiae* significantly reduced mortalities in *E. ictaluri* challenged catfish. We were unable to demonstrate a reduction in *E. ictaluri* associated mortality in β-glucan fed fish. There are two major differences between the present study and that of Chen and Ainsworth (1992). First, fish received β-glucan via the feed in the present study. Presently, there is no evidence that oral administration of β-glucan can be appropriately digested or absorbed and result in a reduction in mortality. Various factors, such as solubility, heat stability, degradation by gastric acid and digestive enzymes, and effective absorption in the gut, determines if a substance such as β-glucan will be effective. Approximately 40% of the β-glucan in the present study is soluble, the remaining β-glucan is insoluble and may be difficult for channel catfish to digest. Channel catfish are capable of utilizing some high molecular weight carbohydrates such as starch and dextrin (Wilson and Poe, 1987); therefore, the absorption of VST® or β-glucan in the gut might be possible. However, whether the VST® is absorbed in an active form is unknown. Many immunostimulants have been studied in fish, yet levamisole (Siwicki, 1989) and EF203 (Yoshida *et al.*, 1993) are the only immunostimulants that have been investigated using oral administration.

Secondly, fish in the present study were exposed to *E. ictaluri* in the water whereas Chen and Ainsworth (1992) administered the bacteria by IP injection. Obviously, injection of the bacteria

into a specific area or region that had previously been primed with β-glucan may result in fewer mortalities. As shown in this study, injection of β-glucan into the peritoneal cavity can elicit neutrophils and activated macrophages which could have an effect on mortality. The weight of catfish used in studies may be a factor in determining the effectivenes of β-glucan. Based on the analysis of the antibody titers in this study, it appears that fish weighing more that 35 g may respond better to β-glucan administration. Fish in the mortality trial were 18 ± 2 g which could, in part, explain the poor response or lack of a response to VST® feeding.

Conversely, in some studies mortalities have been increased in fish administered glucan or other immunostimulants. Robertsen *et al.* (1990) found that fish receiving high concentrations (30-90 μg/g of fish body weight) of M-glucan derived from *S. cerevisiae* occasionally had higher mortality following challenge than control fish. Robertsen (1990) suggests that the higher mortality in fish administered glucan may be due to physically overloading the phagocytes with glucan particles which impairs phagocytic function for 1-2 weeks until the glucan particles are digested. In addition, they found that maximal protection was obtained 3 to 4 weeks after injection of glucan. Similar results, such as increased mortalities or suppression of non-specific defense mechanisms, have been reported by other investigators when using high concentrations of other immunostimulants, e.g. schizophyllan (Yano *et al.*, 1989), levamisole (Anderson, 1992), and Freund's adjuvant (Olivier *et al.*, 1985).

The bacterial concentration used to challenge catfish can also affect the outcome of mortality trials. If catfish are challenged with a high concentration of bacteria they may be overwhelmed and no pretreatment or preventative measures will alter the outcome. If, however, lower concentrations of bacteria are used, then the fish receiving β-glucan rations die at a lower frequency during the first 8 days after challenge than controls (see Table 1). β-Glucan feeding delays the onset of massive mortalities, a similar finding of Chen and Ainsworth (1992). Overdosing with β-glucan, inappropriate timing of the administration of β-glucan, or the size of the fish may have adversely affected the results of the mortality trial. Noted below, in the discussion of the antibody trials is that fish weighing >35g had significantly higher antibody titers; perhaps weight is also a factor in mortality trials. We are presently investigating this possibility.

β-Glucan supplemented feed significantly affected antibody titers of catfish vaccinated to *E. ictaluri*. Rations supplemented with 0.1% β-glucan had a more beneficial effect. Fish fed rations supplemented with 1.0% routinely had antibody titers to *E. ictaluri* that were no different or significantly lower than the controls and 0.1% supplemented groups. Trout fed 0.1% supplemented rations for 6 weeks were immune suppressed (unpublished data) and although the catfish with low antibody titers in this study were not fed the rations for 6 weeks, feeding fish a 1.0% β-glucan supplemented ration for 3 to 4 weeks probably has a similar effect. The weight or size of the fish being fed β-glucan rations also plays a role as to whether a beneficial effect is obtained. Catfish used in the second antibody trial weighed from 17.6 to 70.6 g. Nikl *et al.* (1991) did not demonstrate in fish any effect on antibody titer, however Chen and Ainsworth (1992) demonstrated that β-glucan derived from *Saccharomyces cerevisiae* significantly increased catfish antibody titers to *E. ictaluri*. The results of the present study using 0.1% VST® supplemented rations agree with those of Chen and Ainsworth (1992). The effects of high concentrations of immunostimulants reported elsewhere (Anderson, 1992; Olivier *et al.*, 1985; Yano *et al.*, 1989) correlates well with the effect of high concentrations (1.0%) of β-glucan on antibody titers reported in the present study. Similarly, Anderson *et al.* (1989) and Siwicki and Cossari-Dunier (1990) reported that a high dose of levamisole caused immunosup-

pression by reducing the number of total circulating leukocytes or plaque-forming cells. In the present experiment we did not determine whether the lower serum antibody titer was due to an overdose of VST® causing a reduction in leukocyte number, suppressed phagocytosis, or insufficient antigen processing and presentation. Further studies regarding the relationships between immunostimulants, bacterins, phagocytes, and accessory cells would help to clarify this issue. β-Glucan may be useful in the catfish industry when used alone as a supplement in rations or in conjunction with vaccines because of its ability to increase antibody titers.

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Chapter 8

β-Glucans as Immunostimulants in Fish

Børre Robertsen, Rolf E. Engstad and Jorunn B. Jørgensen

Department of Marine Biochemistry
The Norwegian College of Fishery Science
The University of Tromsø, N-9037 Tromsø, Norway.

ABSTRACT

β-glucans are branched β -1,3- and β–1,6-linked polyglucoses and are major structural polysaccharides in cell walls of most yeasts and mycelial fungi. Higher organisms have developed various recognition mechanisms for these conservative fungal wall components. β-glucans are thus able to stimulate non-specific defence mechanisms in animals as well as plants. It is well established that β-glucans in mammals increase the resistance against microbial infections and stimulate anti-tumour mechanisms. There is now a growing body of evidence that β -glucans also function as immunostimulators in fish. They thus have a potential as feed additives to counteract immunosuppressive states of fish and as vaccine adjuvants.

Soluble β -glucans like scleroglucan, schizophyllan and lentinan have been shown to give protection of carp and yellowtail against bacterial infections and to function as vaccine adjuvants in coho salmon. Our group has demonstrated that a microparticulate β -glucan (M-Glucan, MacroGard®) from the inner cell wall of *Saccharomyces cerevisiae* functions as a potent immunostimulator in Atlantic salmon.

Several lines of evidence show that yeast glucan has a strong ability to stimulate the non-specific defence of fish. Atlantic salmon injected with MacroGard® showed increased resistance to infection by several different bacterial pathogens and increased levels of lysozyme and complement-mediated hemolytic activity in the blood. Yeast glucan has been shown by others to give protection of channel catfish against bacterial infection.

Macrophages appear to be the effector cells of the immunostimulatory activity of yeast glucan in salmonids. Head kidney macrophages from rainbow trout and Atlantic salmon injected intraperitoneally with MacroGard® showed increased killing of *Aeromonas salmonicida* 2 weeks after injection. Furthermore, recent studies have established that MacroGard® has the ability to activate salmon macrophages *in vitro*. Macrophages incubated with 0.1 and 1 μg /mL glucan for 4 to 7 days showed a marked increase in intracellular O_2^- production after exposure to phorbol myristate acetate and opsonized zymosan. MacroGard® also stimulated phagocytosis of glutaraldehyde-fixed SRBC in the macrophages with a peak in activity after 3 days of incubation. MacroGard® has moreover been shown to function as an adjuvant in Atlantic salmon which further confirm its immunostimulatory properties in fish. It increases the protective effect of injectable furunculosis vaccines in

Atlantic salmon and stimulates the specific humoral immune response to the A-layer protein of *A.salmonicida*. This effect may be ascribed to the glucan's ability to attract macrophages to the peritoneum and to stimulate cytokine production in the cells. Recent work show that Atlantic salmon macrophages possess a receptor for yeast β-glucan. Binding to the receptor is possibly important for the immunodulatory effects of the glucan in salmon.

INTRODUCTION

Fish held in aquaculture pens, live in an environment where they are constantly exposed to potential microbial pathogens and may also be carriers of pathogens like IPN virus, *Aeromonas salmonicida* and *Renibacterium salmoninarum*. During their whole life, farmed fish are inevitably subjected to various kinds of stress that may lead to immunosuppression and outbreaks of infections. Common stresses associated with increased susceptibility to disease are handling, transportation, crowding, infections, exposure to pollutants and physiological changes like smoltification. Even though vaccines are available against cold water vibriosis, vibriosis and furunculosis and yersiniosis, Norwegian aquaculture still experiences high losses of the salmon stocks due to diseases. Part of this is due to the fact that even efficient vaccines lose much of their effect one year after vaccination, and that new pathogens, like the ISA virus, are gaining territory.

It seems unlikely that aquacultured fish can be vaccinated against all potential microbial diseases. Also the use of antibiotics and chemical therapeutics in fish farming has to be reduced both to protect the environment and to avoid spreading of antibiotic resistance genes. Stimulation of the non-specific defence is a supplementary method for protection of fish against microbial diseases that is gaining interest in fish farming. A number of different biological and synthetic compounds have been found to increase the natural antimicrobial defence of animals and some of them have shown promising effects in fish (Azuma and Jollès, 1987; Anderson, 1992). Fungi have been shown to be important sources of potent immunostimulants (Di Luzio, 1985). The active compounds are β-glucans which are branched polysaccharides composed of glucose units linked through β-1,3- and β-1,6- glycosidic linkages. The present paper will discuss the immunostimulating properties of β-glucans in fish.

CHARACTERISTICS OF β-GLUCANS

β-glucans are major structural polysaccharides in cell walls of most yeasts, filamentous fungi and mushrooms and are also secreted as a slime or mucilage by some fungi (Rosenberger, 1976). From an evolutionary point of view, these structures probably represent one of the most ancient microbial cell wall components. Consequently, higher organisms may have developed various recognition mechanisms for these conserved fungal wall polysaccharides early in the evolution. This may be the reason why β-glucans have been found to stimulate or activate the non-specific defence mechanisms in a wide range of higher organisms, plants (Darvill and Albersheim, 1984) as well as invertebrates (Unestam and Söderhäll, 1977) and vertebrates (Di Luzio, 1985). On the other hand β-glucans appear to be a poor immunogens in vertebrates (Ballou, 1982).

The immunostimulatory properties of fungal β-glucans were discovered in the sixties by showing their effects against tumor development in mice (DiLuzio, 1985). It has later been demonstrated that β-glucans also increase the resistance of mammals against infection by several bacterial, fungal, viral and protozoal pathogens (Di Luzio, 1985; Di Luzio,1983; Kaneko and Chihara, 1992;

Williams *et al.*,1983). Yeast glucans have also been shown to induce hemopoiesis and to modify various immunosuppressive states in mammals (DiLuzio, 1985). Among the most studied immunostimulatory glucans in mammals are lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune* Fries, scleroglucan from *Sclerotium glucanum* and yeast glucan from *Saccharomyces cerevisiae*. There is now a growing body of evidence that these β-glucans also function as immunostimulators and adjuvants in fish. Scleroglucan, schizophyllan and lentinan are all soluble polyglucoses composed of a β-1,3- linked backbone with single glucosyl branches attached through β-1,6-linkages (Azuma, 1987; Bluhm *et al.*, 1982; Kaneko and Chihara, 1992). They all form a triple helix which is considered important for their biological activity. The first two have branches at every third glucosyl residue in the backbone whereas lentinan has two branches for every five 1,3-linked glucosyl residues (Azuma, 1987; Blume *et al.*, 1982; Kaneko and Chihara, 1992). Lentinan and schizophyllan, have molecular masses of about $5x10^5$d and $4.5x10^5$d. Immunostimulatory yeast glucan is present in the inner cell wall of *S. cerevisiae*. It is microparticulate of nature and insoluble in alkali (Di Luzio *et al.*,1979; Manners *et al.*,1973; Robertsen *et al.*, 1990). The composition of glycosidic linkages in yeast glucan varies with the isolation procedures and is important for its biological activity. Yeast glucan prepared as described by Di Luzio *et al.* (1979) has potent immunostimulatory effects in mice (Di Luzio,1983; Di Luzio *et al.*,1979), but little effect in Atlantic salmon (Robertsen *et al.*, 1990). M-Glucan or MacroGard®, the yeast glucan preparation used as an immunostimulator in Atlantic salmon, is a branched molecule with about 83 % β-1,3-linked glucosyl residues, 5 % β-1,3,6-linked branch points, 6 % β-1,6-linkages and 5 % non-reducing terminals. It exists as particles with a diameter of 1-4 μm (Engstad and Robertsen, 1993). The complete structure of the molecule is not known, but chemical and enzymatic analyses show that it contains long stretches of β-1,3-linked glucose molecules and β–1,3- and β-1,6-linked side chains of various lengths (Manners *et al,* 1973; Engstad and Robertsen, unpublished results). Yeast glucan stripped of β-1,6 side chains, has a molecular mass of about $2.4x10^5$d (Manners *et al.*,1973).

β-glucans potentiate the antibacterial defence of fish

As summerized in Table 1, several lines of evidence show that β-glucans have the ability to increase the non-specific resistance of fish against infections of bacterial pathogens. Intraperitoneal (IP) injection of scleroglucan, schizophyllan and lentinan into carp resulted in enhanced protection against IP infection by the pathogen *Edwardsiella tarda (*Yano *et al.*, 1989, 1991). Schizophyllan and scleroglucan elicited enhanced protection of yellowtail against infection with *Streptococcus* sp. (Matsuyama *et al.*, 1992). Our group has demonstrated that yeast glucan (M-Glucan, MacroGard®) functions as a potent stimulator of the non-specific antibacterial defence in Atlantic salmon. Salmon injected with MacroGard show increased resistance to infection by *Vibrio salmonicida, V. anguillarum, Yersinia ruckeri* and *A. salmonicida* (Robertsen *et al*, 1990 and unpublished results). Glucan treatment of Atlantic salmon gave a relative percentage protection (RPP) of 53-81% in challenge experiments with *V. salmonicida* (Robertsen *et al.*, 1990). Yeast glucan (from Sigma Chemical Co, St. Louis, MO, USA) has recently been shown to give protection of channel catfish against *Edwardsiella ictaluri* (Chen and Ainsworth, 1992).

The non-specific defence is expected to be more effective against opportunistic pathogens than specialized pathogens, and that is indeed what both we and others have observed. In our experiments with Atlantic salmon, injection of yeast glucan gave better protection against *V. salmonicida* than *V. anguillarum* and least protection against *A. salmonicida* (Robertsen *et al.*, 1990 and unpublished

Table 1
Reports of enhancement of resistance in fish against bacterial infections with β-glucans

Glucan	Administration (Dose)	Fish Species	Pathogen	References
Lentinan Schizophyllan Scleroglucan	IP-injection (2-10 mg/kg)	Carp	*E. tarda* *A. hydrophila*	Yano *et al.*, 1989 Yano *et al.*, 1991
Schizophyllan Scleroglucan	IP-injection (2-10 mg/kg)	Yellowtail	*Streptococcus sp.*	Matsuyama *et al.*, 1992
Yeast glucan	IP-injection (2-100 mg/kg)	Atlantic salmon	*V. anguillarum* *V. salmonicida* *Y. ruckeri*	Robertsen *et al.*, 1990
Yeast glucan	Oral (0.1-1% in feed)	Atlantic salmon	*V. salmonicida* *V. anguillarum*	Raa *et al.*, 1992
Yeast glucan	IP-injection (0.5-0.7 mg/kg)	Channel catfish	*E. ictaluri*	Chen and Ainsworth, 1992
VitaStim-Taito®	IP-injection (20 mg/kg)	Coho salmon	*A. salmonicida*	Nikl *et al.*, 1991
VitaStim-Taito®	Oral (0.1-1% in feed)	Chinook salmon	*A. salmonicida*	Nikl et al.

results). Matsuyama *et al.* (1992) observed that injection of glucans into yellowtail gave protection against *Streptococcus* sp., but not against *Pasteurella piscicida*, a pathogen which replicates in phagocytic cells.

The duration of protection obtained with glucans has been given little attention so far. In the experiments with scleroglucan, schizophyllan and lentinan, single or double injections of 2-10 mg/kg were given and challenge performed (Matsuyama *et al.*, 1992; Yano *et al.*, 1989, 1991). Our studies with Atlantic salmon showed that injection of low doses of glucan (2-10 mg/kg) gave protection at day 7 after injection whereafter the protection declined. High doses (100 mg/kg) gave no protection 1 week after injection and maximal protection 3-4 weeks after injection whereafter the protection declined (Robertsen *et al*, 1990). A limited duration of protection is expected when the non-specific defence is triggered with a single injection of a metabolizable stimulus.

Oral administration of glucans over a longer period of time may give prolonged protection against microbial infections, but such studies have not yet been published. However, data have been presented which indicate that glucans incorporated in the feed really are able to enhance the resistance of fish against pathogenic bacteria (Nikl *et al.*, 1992; Raa *et al.*, 1992).

Table 2
Non-specific defense mechanisms elicited by β–glucans in live fish

Enhanced activity	Fish species	Glucan type[a]	References
Lysozyme activity	Atlantic salmon	YG	Engstad *et al.*, 1992
	Rainbow trout	YG	Jørgensen *et al.*, 1993b
	Yellow tail	SG, SP	Matsuyama *et al.*, 1992
Complement activity	Atlantic salmon	YG	Engstad *et al.*, 1992
	Yellowtail	SG, SP	Matsuyama *et al.*, 1992
Phagocytic activity of head kidney cells	Carp	L, SG, SP	Yano *et al.*, 1989
	Channel catfish	YG	Chen and Ainsworth, 1992
Bacterial activity of head kidney cells	Channel catfish	YG	Chen and Ainsworth, 1992
Bactericidal activity of head kidney macrophages	Rainbow trout	YG	Jørgensen *et al.*, 1993b
	Atlantic salmon	YG	Jørgensen *et al.*, 1993a

[a]L=lentinan, SG=scleroglucan, SP=schizophyllan, YG=yeast glucan

Table 2 shows a summary of cellular and noncellular defence mechanisms which increase in activity after treatment of fish with β-glucans.

Non-cellular components

The non-specific defence of fish consists of phagocytic cells (neutrophils and macrophages) and a range of antimicrobial proteins or glycoproteins in tissues and body fluids. The non-cellular, non-specific defence includes metal ion chelators which inhibit microbial growth (transferrin, ceruloplasmin, metallothionein), interferons, protease-inhibitors (α-2 macroglobulin and other α-globins), lytic enzymes (lysozyme, chitinase, nonspecific lysins), complement, lectins, C-reactive protein and other precipitins and mucopolysaccharides (Alexander and Ingram, 1992). Only a few of these factors have so far been studied in relation to the mode of action of glucans or other immunostimulants in fish. Nevertheless, it has been demonstrated that injection of yeast glucan into Atlantic salmon (Engstad *et al.*, 1992) and schizophyllan and scleroglucan into yellowtail (Matsuyama *et al.*, 1992) resulted in enhanced lysozyme and complement-mediated hemolytic activity in the fish blood (Figures 1 and 2).

Purified rainbow trout lysozyme is reported to have antibacterial activity against several Gram-negative fish pathogenic bacteria (Grinde, 1989), indicating that elevated lysozyme-levels could contribute to enhanced destruction of invading bacteria. Increased complement activity may give increased phagocytosis of bacteria or direct killing of bacteria. β-glucans have also been shown to give increased lysozyme and complement activity in mice (Kokoshis and Di Luzio, 1979; Sakamoto *et al.*, 1983). We did not observe increases in other lysosomal enzymes in Atlantic salmon blood, which indicates that the increase in lysozyme activity is selective and not due to disintegration or degranulation of phagocytic cells (Engstad *et al.*, 1992). On the other hand the increased activity of lysozyme and complement could be due to increased synthesis of these components in mononu-

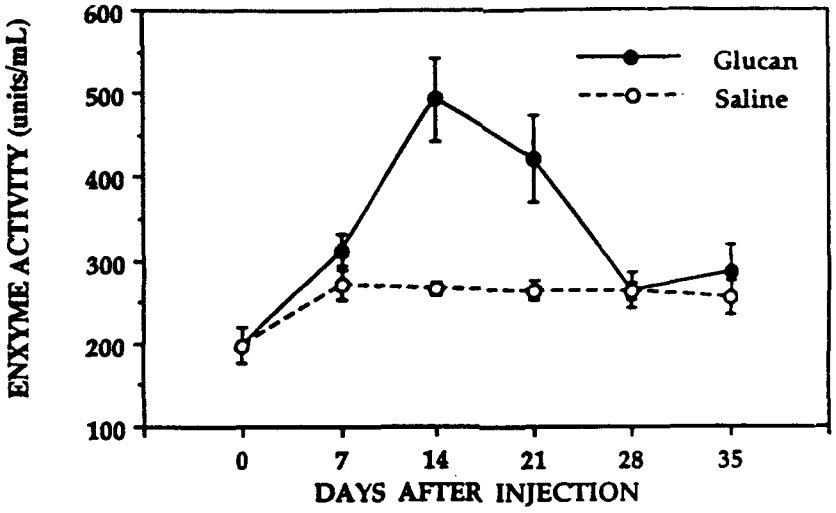

Figure 1. Lysozyme activity in plasma from Atlantic salmon after injection of M-Glucan or saline. Fish with an average weight of 150 g were IP-injected with 1 mL suspension of M-Glucan (10 mg/mL, w/v) in saline or 1 mL saline (control). Day 0 is activity prior to injection. Each point represent the mean activity S.E. in plasma from 10 fish. Adapted from Engstad *et al.*, 1992.

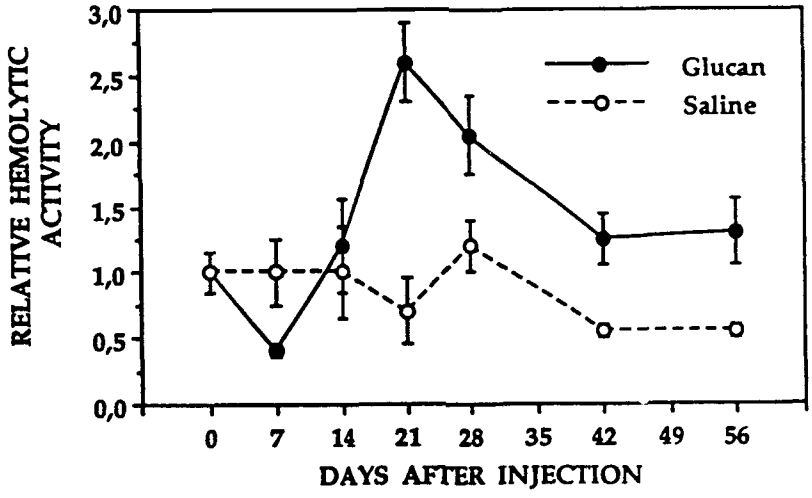

Figure 2. Complement-mediated hemolytic activity against SRBC in plasma from Atlantic salmon after IP-injection of M-Glucan or saline. Fish with an average weight of 80 g were IP-injected with a 0.7 mL suspension of M-Glucan (10 mg/mL, w/v) or 0.7 mL saline. The data are presented as relative hemolytic activity to day 0. Each point represent the mean activity S.E. in plasma from 10 fish. Adapted from Engstad *et al.*, 1992.

clear phagocytes or increased production of mononuclear phagocytes themselves (Engstad *et al.*, 1992; Kokoshis and Di Luzio ,1979).

β-glucans are in general also potent activators of the alternative complement pathway both in fish and mammals (Czop and Austen, 1985b; Yano *et al.*, 1989, 1991). This reaction may result in an initial consumption of complement and the generation of complement components which function as chemoattractants (C5a) and activators of phagocytes (C3a and C5a). The importance of this reaction for immunostimulation is not known either for fish or mammals although activation of complement was not found to be important for the glucan-induced protection of mice against septic shock (Williams *et al.*, 1988b).

Phagocytes

Because many bacterial pathogens have developed surface components that prevent the opsonic or bactericidal activity of complement and lysozyme, it is generally believed that phagocytic cells are more important in immunostimulation.

Several studies show that intraperitoneal (IP) injection of β-glucans into fish have a stimulatory effect on phagocytic cells. Pronephric cells of both yellowtail, channel catfish and carp showed enhanced phagocytic activity after IP injection of soluble β-glucans (Matsuyama *et al.*, 1992; Yano *et al.*, 1989; Chen and Ainsworth, 1992). As shown in Figure 3, IP injection of yeast glucan into rainbow trout elicited enhanced bactericidal activity of head kidney macrophages against *A. salmonicida* (Jørgensen *et al.*, 1993b) and a similar effect was shown for Atlantic salmon (Jørgensen *et al.*,1993a). The increased bactericidal activity of macrophages from trout, but not salmon, was accompanied by an augmentation in respiratory burst activity. Chen and Ainsworth (1992) observed enhanced phagocytic and bactericidal activity of head kidney cells, mostly neutrophils, after

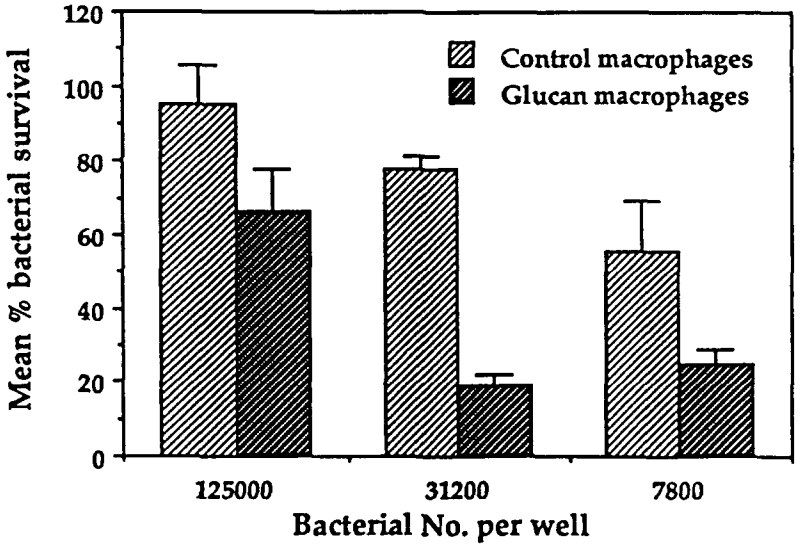

Figure 3. Mean percentage survival of *A. salmonicida* strain MT 423 incubated with rainbow trout head kidney macrophages isolated from M-Glucan injected fish (glucan macrophages) or saline injected fish (control macrophages) 2 weeks post injection (±. S.E., N=5). Adapted from (Jørgensen *et al.*, 1993b).

glucan-treatment of channel catfish. The cells did, however, not show increased production of hydrogen peroxide (Chen and Ainsworth, 1992).

The peritoneal and head kidney cell response of Atlantic salmon after IP injection of yeast glucan was recently studied (Jørgensen *et al.*,1993a). Injection of glucan resulted in accumulation of macrophages, neutrophils and thrombocytes in the peritoneum with a maximum response after 2 days. The number of leukocytes then declined rapidly, but settled at a level which was 5-10 times higher than in the control fish. This effect may be important both for the stimulation of the non-specific defence by the glucan and for its effect as an adjuvant (see below). The migration of leukocytes may be mediated by chemoattractants that in part are generated by glucan-mediated activation of the complement (C5a) and in part by eicosanoids (leukotriene B_4 and lipoxin A_4) released from macrophages (Secombes and Fletcher, 1992). The glucan-treatment resulted in an elevated number of neutrophils in the head kidney 3 weeks after injection, but the number of other head kidney leukocytes did not change (Jørgensen *et al.*,1993a). Altogether these reports demonstrate that IP injection of glucans into fish have both systemic and local effects on the non-specific defence.

ACTIVATION OF ATLANTIC SALMON MACROPHAGES WITH YEAST GLUCAN *IN VITRO*

Because activation of macrophages appeared to be involved in the glucan-induced antibacterial defence of live salmonid fish, we decided to study the effect of yeast glucan on head kidney macrophages from Atlantic salmon *in vitro*. Salmon macrophages were incubated for 1-7 days with various concentrations of yeast glucan (MacroGard®) in 96 well culture plates and tested for respiratory burst activity (NBT-assay) after exposure to phorbol myristate acetate (PMA) or opsonized zymosan. The macrophages were also tested for phagocytic activity against glutaraldehyde-fixed sheep red blood cells (SRBC) and bactericidal activity against a virulent (MT 423) and an avirulent (MT 004) strain of *A. salmonicida* (Jørgensen, J.B. & Robertsen, B., unpublished results). The macrophages showed a marked increase in respiratory burst activity 4 to 7 days after addition of 1 µg/mL glucan. Interestingly, the increase in respiratory burst activity of glucan-treated macrophages showed a maximum at glucan-concentrations of 0.1-1 µg/mL whereas 10 µg/mL had no effect and 50 µg/mL was inhibitory (Figure 4).

Glucan-stimulated macrophages also showed an increase in phagocytic activity against SRBC which reached a maximum after 3 days of incubation and thus appeared to occur earlier than the increase in respiratory burst activity. These results demonstrate that yeast glucan is able to activate important antimicrobial mechanisms in Atlantic salmon macrophages. The respiratory burst data also indicate that high concentrations of glucan may induce the production of self-inhibitory compunds in the macrophages or be toxic for them. These observations suggest that use of glucans for immunostimulatory purposes in applied aquaculture should be tested out in a range of different concentrations to find the optimal concentration regime.

Despite the stimulatory effect of glucan on phagocytosis and respiratory burst activity of Atlantic salmon macrophages, the macrophages did not show increased bactericidal activity against the avirulent and virulent strain of *A. salmonicida*. This shows that upregulation of these anti-microbial functions alone is not sufficient to give Atlantic salmon macrophages the ability to kill *A. salmonicida*. Whether less specialized pathogens are killed by glucan- treated macrophages is not yet known.

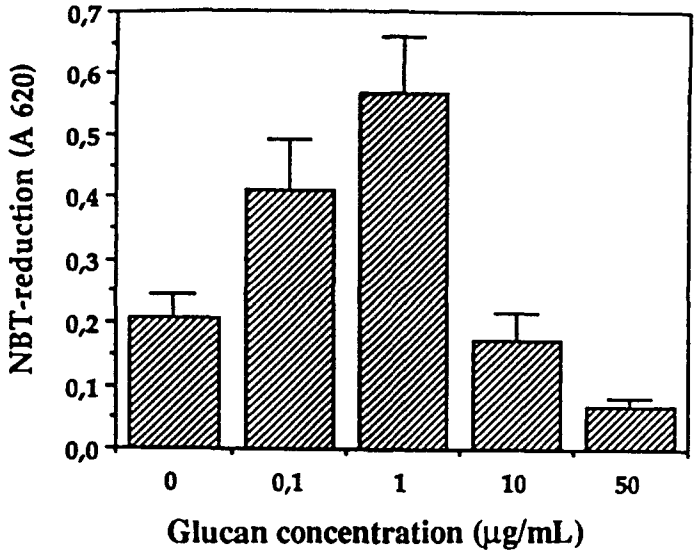

Figure 4. Respiratory burst activity of head kidney macrophages from Atlantic salmon cultured for 7 days in the presence of various concentrations of M-Glucan *in vitro*. Respiratory burst activity was measured as the reduction of nitroblue tetrazolium (NBT) after addition of PMA (200 ng/mL). Data are given as mean absorbance at 620 nm per 10^5 cells (±. S.E., N=4).

The role of cytokines in immunostimulation by β-glucans

The *in vitro* studies indicated that yeast glucan has the ability to activate selected antibacterial functions of Atlantic salmon macrophages, but not a full capacity to kill virulent *A. salmonicida* bacteria. The increased killing of *A. salmonicida* expressed by head kidney macrophages from glucan-treated rainbow trout and Atlantic salmon (Jørgensen *et al.*, 1993a; 1993b), may thus be due to the co-influence of lymphokines. Graham and Secombes (1988) have previously demonstrated that a macrophage activating factor (MAF) is produced by rainbow trout leukocytes stimulated with ConA. Trout macrophages treated with MAF showed not only increased respiratory burst activity, but also bactericidal activity against a virulent strain of *A. salmonicida* (Graham and Secombes 1988). Whether MAF is involved in the antibacterial resistance induced by glucans or other immunostimulants is an important issue of future studies.

Mammalian macrophages produce cytokines that may activate lymphocytes (interleukin 1, IL-1) or themselves (tumor necrosis factor-α, TNF). IL-1 has been shown to potentiate both the specific and the non-specific defence of mice possibly due to its ability to activate T-lymphocytes (Cheers *et al.*, 1990). Both yeast glucan and lentinan elicit the production of IL-1 in mammalian mononuclear phagocytes (Abel and Czop, 1992; Fruehauf *et al.*, 1982). IL-1 like molecules have recently been demonstrated in channel catfish (Ellsaesser, 1989). Preliminary results by Jørgensen, J.B., Rasmussen, L., Seljelid, R. & Robertsen, B. at the University of Tromsø, using a mouse thymocyte proliferation assay, indicate that Atlantic salmon macrophages also produce IL-1 like molecules *in vitro* and that yeast glucan enhances the production of such molecules (Figure 5).

TNF has been shown to be important in the antibacterial defence of mice (Havell, 1989) and it has been shown that yeast glucan elicits production of TNF in monocytes (Abel and Czop, 1992). Production of TNF by fish macrophages has to our knowledge not yet been shown. However, Hardie

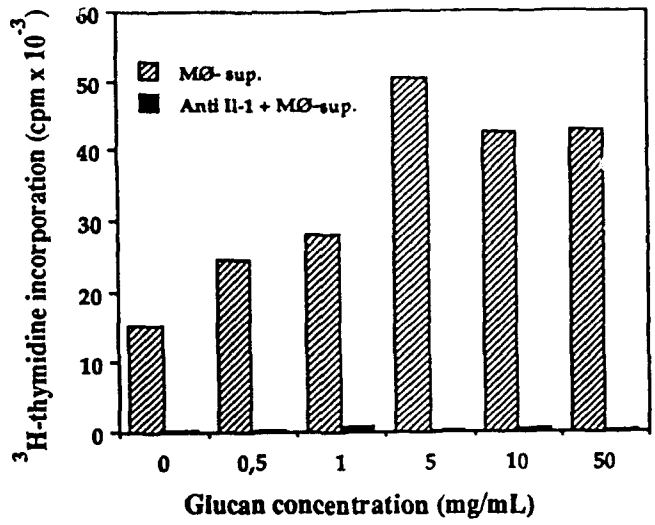

Figure 5. Proliferative responses of mouse thymocytes to salmon macrophage supernatants to which ConA is added. Head kidney macrophages from Atlantic salmon were cultured *in vitro* in the presence of M-Glucan at various concentrations for 48 hr. Macrophage supernatants were then added to mouse thymocytes in the presence of ConA (0.5 µg/mL). One series of supernatants were treated with rabbit anti-human IL-1 at a dilution of 1: 50 prior to addition to thymocytes. Incorporation of ^{3}H-thymidine into thymocytes was assayed by standard methods. Data represent the mean cpm of 2 thymocyte cultures.

et al. (1993) recently reported that human recombinant TNF synergised with MAF to elevate respiratory burst activity of rainbow trout macrophages which indicate the presence of TNF receptors in these cells.

Recent experiments in our laboratory showed that a polyclonal rabbit antiserum against human recombinant TNF was able to reduce the respiratory burst activity of isolated Atlantic salmon macrophages which were activated with yeast glucan (Figure 6, Jørgensen, J.B. & Robertsen,B.,

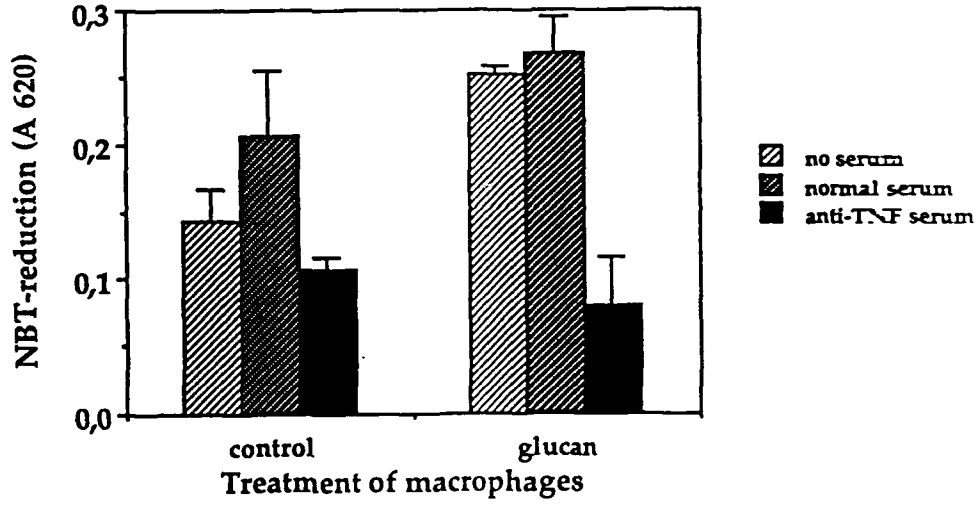

Figure 6. Effect of antibodies against TNF on the respiratory burst activity induced by yeast glucan in Atlantic salmon macrophages. Head kidney macrophages were cultured *in vitro* in the absence(control) or in the presence of M-Glucan (1 µg/mL) for 4 days. To some cultures was added normal rabbit serum and to some, a polyclonal rabbit anti-human TNF-α serum (1: 50 dilutions). Respiratory burst activity was assayed as described in Figure 4.

unpublished results). Normal rabbit serum and rabbit antiserum against human IL-1 did not reduce the glucan-induced respiratory burst activity of the macrophages. This suggests a role for TNF in the activation of Atlantic salmon macrophages by yeast glucan.

β-GLUCANS FUNCTIONS AS ADJUVANTS IN FISH VACCINES

Some immunostimulatory compounds have the ability to function as adjuvants which means that they augment the specific immune response of an animal to antigens. The classical adjuvant is *Mycobacterium* bacteria in which muramyl dipeptide is the most important active principle. Adjuvant activity of β-glucans have been shown in mammals (Williams *et al.*, 1989) and have recently been demonstrated in fish both by our laboratory (Rørstad *et al.*, 1993) and Nikl and coworkers (1991) using *A. salmonicida* bacterins as the vaccine component. The latter group also included Freunds incomplete adjuvant in their mixtures. Nikl *et al.* (1991) observed enhanced protection of fish that were vaccinated with bacterin together with lentinan or Vita Stim-Taito®, a β-glucan extracted from mycelia of *S. commune*, compared to fish vaccinated with bacterin alone. They were, however, unable to detect effects of the glucans on antibody-production against *A. salmonicida*. Rørstad *et al.* (1993) showed that yeast glucan (MacroGard®) increases the protective effect of injectable furunculosis vaccines in Atlantic salmon (Figure 7). It was established that the

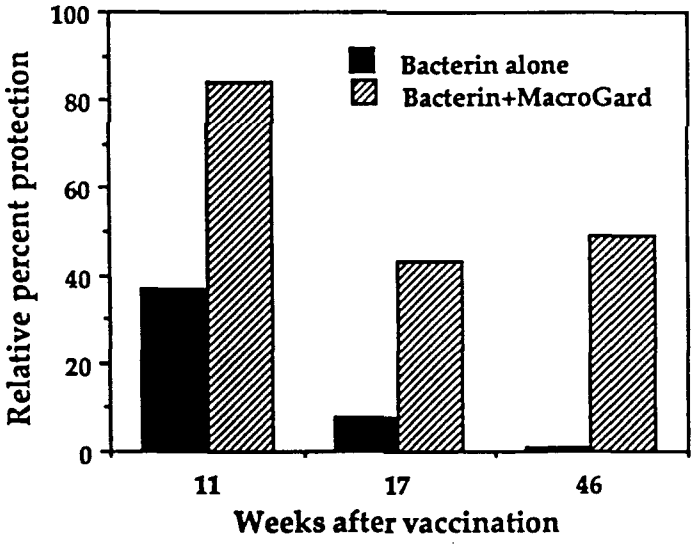

Figure 7. Protection of Atlantic salmon presmolts vaccinated with an *A. salmonicida* bacterin alone or bacterin added MacroGard®. The fish were challenged after various time intervals using a cohabitation challenge method. (Courtesy of Dr. Per Martin Aasjord).

glucan stimulates the specific humoral immune response of the fish to the A-layer protein of *A. salmonicida* , but not to the lipopolysaccharide or extracellular proteins of the bacterium (Rørstad *et al.*, 1993, Figure 8). These results indicate that the glucan does not have an effect on B-lymphocytes, but that it may stimulate T-cell functions or antigen-presentation of bacterial surface protein(s). The latter is supported by the observation that IP injection of yeast glucan into Atlantic salmon results in accumulation of macrophages into the peritoneum 2 days later (Jørgensen *et al.*, 1993a). Macrophages are known to be important antigen-presenting cells of vertebrates including fish. Stimulation of the production of IL-1 like molecules in macrophages may also contribute to

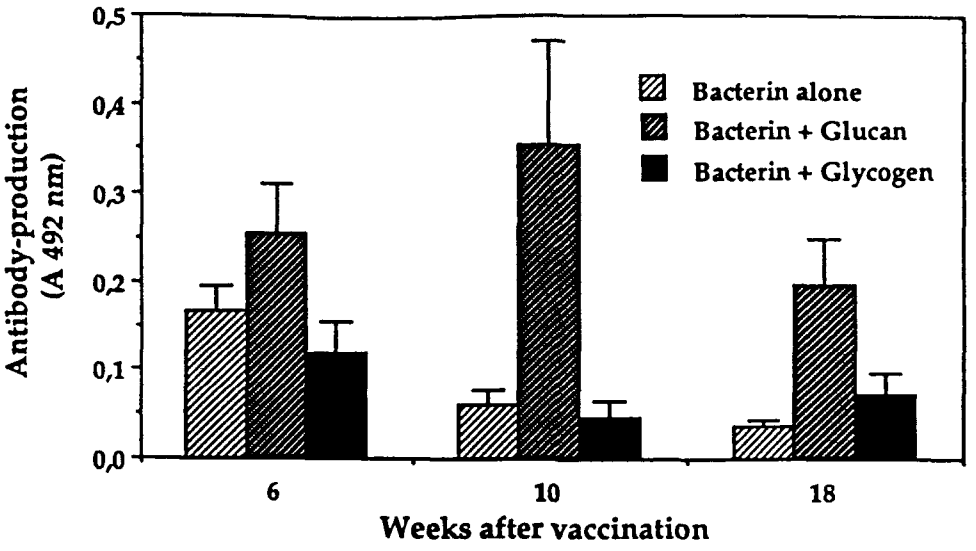

Figure 8. Adjuvant effect of M-Glucan on the humoral immune response of Atlantic salmon against the A-layer protein of *A. salmonicida*. Atlantic salmon premolts were vaccinated with a whole cell *A.salmonicida* bacterin alone, bacterin added M-Glucan or bacterin added glycogen. Sera were collected after 6, 10 and 18 weeks and assayed for antibodies against A-layer protein using an ELISA method. Data are the mean ± SE for 10 individuals.

the adjuvant effect. Whether the protective effect of glucan is due to the increased humoral response to the A-layer protein or stimulation of the cellular immunity is not yet known. β-glucans apparently also have adjuvant properties in channel catfish because injection of channel catfish with yeast glucan and heat-killed *E. ictaluri* in Freund's incomplete adjuvant on the same day, resulted in enhanced antibody-production against the bacterium (Chen and Ainsworth, 1992).

Atlantic salmon macrophages have a specific receptor for yeast glucan

It has been established that both human mononuclear phagocytes and mouse macrophages have a specific receptor for yeast glucan and the former has been purified (Czop and Kay, 1991; Goldman, 1988). Phagocytosis of yeast glucan by human monocytes through the glucan receptor elicits a range of responses that may have a role in immunostimulation (Abel and Czop, 1992; Czop and Austen, 1985a). Our laboratory has recently demonstrated that Atlantic salmon macrophages most likey also possess a specific receptor for yeast glucan because the phagocytosis of glucan particles is specifically inhibited by soluble β-1,3-linked glucans (Engstad and Robertsen, 1993). Activation of salmon macrophages *in vitro* by yeast β-glucan, may thus occur by stimulation of the β-glucan receptor. Whether stimulation of the non-specific defence of vertebrates *in vivo* occurs through β-glucan receptors on mononuclear phagocytes is still uncertain because most immunostimulatory β-glucans are also powerful activators of the alternative complement pathway in both fish and mammals. They may thus be phagocytosed through complement receptors as well as glucan receptors.

CONCLUSION

Work done in several laboratories shows that β-glucans have a great potential as immunostimulants in fish aquaculture. They may be used both to counteract immunosuppressive states of fish and as vaccine adjuvants. Some glucans have in fact already been applied commercially as fish feed additives and adjuvants in furunculosis vaccines. However, there is a definite need for further research on the mode of action of glucans in the fish as well as on applied aspects.

Even with respect to mammals, it is not known how β-glucans stimulate the non-specific defence despite the availability of pure cytokines, antibodies against cytokines and methods to obtain pure leukocytes and T cell deficient animals. This is probably due to the complex network of signals that occur between the different leukocytes during immunostimulation. The principal target and effector cell for the immunostimulatory effects of glucans in mammals is, however, thought to be the macrophage (Di Luzio, 1985; Kaneko and Chihara, 1992; Seljelid *et al.*, 1985; Williams *et al.*, 1983). This is due to the fact that macrophages possess specific receptors for β-glucans and have a key regulatory role in the immune system of vertebrates. Macrophages are not only "professional" phagocytes, but regulate the function of lymphocytes and themselves through the production of interleukins, TNF, leukotrienes and prostaglandins. They also produce lysozyme and complement components. Phagocytosis of yeast glucan particles through the glucan receptor elicits a range of responses in human monocytes including the generation of leukotrienes (Czop and Austen, 1985a) and production of TNF and IL-1b (Abel and Czop, 1992). Treatment of rats with a soluble yeast glucan increased the plasma and splenic levels of IL-1 and IL-2 which suggests a role for these cytokines in the anti-infectivity of glucans (Sherwood *et al.*, 1987). Lentinan also elicits IL-1 production in murine macrophages (Fruehauf, *et al.*, 1982). Besides the importance of macrophages, lentinan is known to be a T-cell oriented immunostimulant and some of its effects in mammals are dependent on B-cells and natural killer cells. In addition to the participation of macrophages, the protective role of yeast glucan in mice appears to be mediated in part by increased production of neutrophils (Williams *et al.*, 1988).

The enhanced resistance of fish against bacterial infections obtained with β-glucans correlates with increased activities of blood lysozyme and complement, phagocytic activity of head kidney cells and bactericidal activity of head kidney macrophages.

The demonstration of *in vitro* activation of respiratory burst and phagocytic activity of Atlantic salmon macrophages by yeast glucan, support that activated macrophages have a role in the glucan-induced defence. The observation that macrophages from glucan-treated fish, but not glucan-treated macrophages, showed increased bactericidal activity against *A. salmonicida* suggests that lymphokines or other endogenous mediators may be involved in macrophage activation *in vivo*. As discussed above, we therefore think that the role of MAF in glucan-stimulation of fish should be examined more closely.

Preliminary evidence from *in vitro* studies suggests a role for IL-1- and TNF-like molecules as mediators in the immunostimulation of Atlantic salmon by yeast glucan and these molecules also deserve more attention. Obviously the progress in elucidation of mechanism of action of immunostimulants in fish is dependent on the progress in research which deals with the purification of fish cytokines, the making of antibodies against them and the purification of T lymphocytes.

On the applied side, future studies should focus on the effect of β-glucans on the resistance of fish against virus and parasites. More studies are also needed on the stimulation of the non-specific defence after oral administration of β-glucans in fish. Duration of protection and optimal stimulation regimes have to be defined and challenge experiments with a wider range of pathogens have to be carried out. It is also of great importance to study if β-glucans are taken up from the fish gut, what kind of gut cells that the glucans encounter first and their effects on gut leukocytes (attraction of phagocytes, activation of macrophages,etc.).

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Chapter 9

Immunological Alterations as Indicators of Environmental Metal Exposure

Judith T. Zelikoff

Institute of Environmental Medicine
New York University Medical Center
New York, New York, 10016 USA

ABSTRACT

While cadmium represents a major aquatic pollutant in many parts of the world, and is carcinogenic and immunotoxic in a variety of mammalian species, relatively little is known concerning the effects (and underlying mechanisms) of cadmium exposure on fish immunocompetence. This chapter reviews the present literature on cadmium-induced immunotoxicity in fish and describes results obtained in our laboratory with rainbow trout (*Oncorhynchus mykiss*) that support and expand the limited current knowledge. There is also a discussion of the applicability of macrophage functional assays (described in this chapter) to serve as indicators of the immunotoxicological effects of low level cadmium exposure on feral fish populations.

BACKGROUND

Immune defense mechanisms in fish have not been as extensively studied as those of mammals, but they share a number of structural and functional characteristics important to the humoral, cell-mediated, and nonspecific immune responses (reviewed in Zelikoff, 1993; Enane *et al.* 1993). For example, fish can reject allografts, exhibit hypersensitivity responses, synthesize specific antibodies following antigenic stimulation, and release biological response modifiers, including cytokines, arachidonic acid metabolites, and cytokines. Because of these overlapping characteristics, there is increasing interest in the immune responses of fish as models for higher vertebrates in immunotoxicological studies (Zelikoff *et al.* 1991) and as indicators of the effects of environmental pollutants (Weeks and Warinner, 1988; Bowser *et al.* in press).

While the effects of metal toxicants upon immune defense mechanisms in mammals have been well-studied, only a limited number of *in vitro /in vivo* laboratory studies have been performed to examine metal-induced immunomodulation in fish. As a result, relatively little is known concerning the immunotoxic effects of metal contaminants in fish (Robohm and Nitkowski, 1976; Zeeman and Brindley 1981; O'Neill, 1981a,b; Viale and Calamari, 1984; Elasser *et al.* 1986; Cossarini-Dunier, 1987; Cossarini-Dunier *et al.* 1988; Thuvander, 1989; Zelikoff, 1993; Bowser *et al.* in press). This chapter reviews the immunotoxicity of cadmium (Cd) in higher teleosts and describes results obtained in our laboratory that support and expand the limited current knowledge. The applicability

of the macrophage functional assays decribed to serve as indicators of the immunotoxicological effects of low level Cd exposure on feral fish populations will also be discussed.

INTRODUCTION

Fish are exposed to toxic chemicals mainly from discharges to rivers, lakes, and oceans as well as from marine dumping, and atmospheric fallout. In the North Atlantic ecosystem alone, hundreds of pollutant chemicals have been identified and quantified (Meyerson *et al.* 1981; O'Connor and Huggett, 1987). These chemicals include metals and metalloids, as well as synthetic and chlorinated organics, and polycyclic aromatic hydrocarbons (PAHs) (Meyerson *et al.* 1981; MacLeod *et al.* 1981; O'Connor and Huggett, 1987). For example, mean concentrations as high as 400 ppm of lead (Pb), mercury (Hg), and Cd have been measured in Newark Bay sediments (Meyerson *et al.* 1981). In Puget Sound, arsenic (As), Pb, and Hg concentrations are significantly elevated in the urban embayments of Sinclair Inlet and Commencement and Elliott Bays (compared to nonurban reference areas) (Malins *et al.* 1984). In other parts of the world, levels of toxic heavy metals over and above those found naturally in the aquatic environment have been measured in the water, sediment, and/or in the tissues of those organisms that reside there. For example, perch (*Perca fluviatilis*) living in the Cd-contaminated river Emän in southeastern Sweden have liver Cd levels 6-8 times higher than those measured in reference perch (Sjobeck *et al.* 1984). Fish taken from the North Sea and distant-water fishing grounds (in the vicinity of England and Wales) have body burdens of Pb 8 to 12-fold above government regulated limits (0.5 ppm Pb) (Portmann, 1972). Furthermore, some commercial species of New Zealand sea fish have liver concentrations of Cd as high as 54 ppm Cd (Brook and Rumsey, 1974).

Municipal wastes, industrial discharges, surface runoff, damage to and weathering of vessel protective paints, ocean dumping, and aerial inputs account for most ocean metal pollution. Weathering accounts for almost one half of the yearly input of Hg into the environment. The current interest in mineral mining, energy development and use, and dredging will undoubtedly result in further metal pollution of aquatic environments. Some of the routes of entry for metals into the oceans have been limited or circumvented in recent years, but in many cases regulation has been implemented too late.

The growing environmental pollution by potentially toxic metals gives rise to particular problems in the aquatic environment. The constant contact with, and reactivity of these metal pollutants on aquatic organisms may result in bioaccumulation. Metal accumulation is dangerous not only for the survival of the directly exposed organism, but also for humans that may consume these species. The effects of metal pollution are measurable on both ecological and economic scales. Ecosystem impacts include contamination of sediments and the water column, accumulation of pollutants in biota over a wide area, and apparent increases in pollutant-related anomalies in the species that reside there.

The biological effects of metallic pollutants such as As, Cd, and Pb in aquatic environments are significant. Documented effects include alterations in hematological parameters, enzyme functions and/or gene expression, homeostasis, carbohydrate metabolism, embryonic and/or ova development, and immunological competence (Larsson, 1975; Johannson-Sjobeck and Larsson, 1979; Larsson *et al.* 1981; Weis *et al.* 1981; Lehtinen *et al.* 1985; O'Connor and Huggett, 1987; Weeks and Warriner, 1988; Smith, 1990; Ghanmi *et al.* 1990; Zelikoff, 1993; Bowser *et al.* in press).

Effects on aquatic life may be estimated from these biological responses to heavy metal exposure, as well as from the incidence of pollution-related disease in these exposed organisms.

Cadmium-induced immunomodulation

Cadmium represents a major aquatic pollutant in many parts of the world (Brooks and Rumsey, 1974; Sjobeck *et al.* 1984). Although Cd accumulates in high concentrations in fish, and despite its widespread distribution in the aquatic environment, its carcinogenicity in rodent models (and, possibly, in humans), and its potent immunotoxicity in a variety of mammalian species (Muller *et al.* 1979; Koller and Vos, 1981; Bozelka and Burkholder, 1982; Waseem *et al.* 1993), relatively few studies have examined its effects on fish immune defense mechanisms (Robohm and Nitkowki, 1976; O'Neill, 1981a,b; Viale and Calamari, 1984; Elasser *et al.* 1986; Thuvander, 1989; Enane *et al.* 1991; Enane, 1991). As a result, little is known concerning the effects and underlying mechanisms of Cd exposure on fish immunocompetence.

Exposure to Cd *in vitro* dose-dependently alters the chemiluminescent response of carp (*Cyprinus carpio*) pronephric macrophages in response to *Staphylococcus aureus* (Elasser *et al.* 1986). Enane *et al.* (1991) has demonstrated that *in vitro* exposure to relatively non-toxic concentrations of $CdCl_2$ alters resident and *Aeromonas salmonicida*-activated peritoneal macrophage functional activities in rainbow trout (*Oncorhynchus mykiss*). The authors demonstrate that exposure of trout macrophages to Cd enhances phagocytosis as well as zymosan-stimulated production of hydrogen peroxide (H_2O_2). They suggest that the observed increase in H_2O_2 production is a result of either: (a) the increased interactions of superoxide with the cysteinyl thiolate groups of Cd-induced metallothionein leading, ultimately, to the enhanced production of thiol radicals and of H_2O_2 (Thornalley and Vasak, 1985); or to (b) direct inhibition by Cd of the iron-mediated non-enzymatic reduction of H_2O_2 to hydroxyl radicals (Thomas *et al.* 1986). The latter process would decrease H_2O_2 degradation and result in its prolonged presence in the system both at the intracellular and extracellular level. The authors conclude, however, that the mechanisms involving metal ions and active oxygen species are complex and that data supporting these hypotheses are limited.

The data on the effects of *in vivo* Cd exposure upon fish immunocompetence are also scant and somewhat contradictory (Robohom and Nitkowski, 1976; O'Neill, 1981a; Viale and Calamari, 1984; Robohm, 1986; Thuvander, 1989). For example, Thuvander (1989) demonstrated that waterbath exposure of rainbow trout to Cd for 12 weeks enhanced their antibody response to *Vibrio anguillarum*. In contrast, however, Viale and Calamari (1984), report a slight reduction in antibody production (compared to the control) against human red blood cells following a 4 month waterbath exposure of rainbow trout to Cd. In addition, intraperitoneal (IP) administration of Cd depressed antibody levels (in a dose-dependent fashion) in brown trout inoculated with *MS2* bacteriophage (O'Neill, 1981b).

In addition to its effects on humoral immunity, *in vivo* Cd exposure alters specific cell- and macrophage-mediated immune responses of fish (Thuvander, 1989). For example, waterbath exposure of *V. anguillarum*-injected rainbow trout to Cd reduces the lymphoproliferation of recovered splenocytes in response to mitogens and to an extract of *V. anguillarum* antigen (Thuvander, 1989). Robohm (1986) observed that peritoneal macrophages from striped bass exposed to Cd show enhanced migration through microporous filters, and Robohm and Nitkowski (1976) demonstrated that waterbath exposure of cunners (*Tautoglabrus adsperus*) to Cd increases

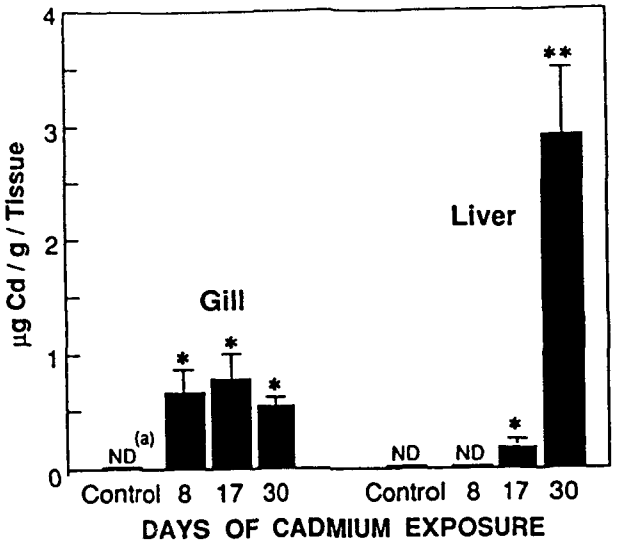

Figure 1. Body burdens of Cd in rainbow trout after 8, 17, and 30 days of Cd exposure at 2 ppb. Non-detectable (ND) limit (a) by atomic absorption spectrophotometry for liver is 0.125 µg/g and for gills 0.5 µg/g tissue. Cadmium levels in the gills were significantly increased (*p <0.05) above control gill values after 8, 17, and 30 days of exposure. Cadmium levels in the liver increased with increasing Cd exposure time; after 17 days of exposure, Cd levels were significantly enhanced (*p <0.05) above unexposed control tissue levels and 8 day exposure values. After 30 days of exposure, Cd levels in the liver of exposed trout were significantly increased (**p <0.001) above the levels in fish exposed for 17 days. Values represent the mean ±SE of 6 fish.

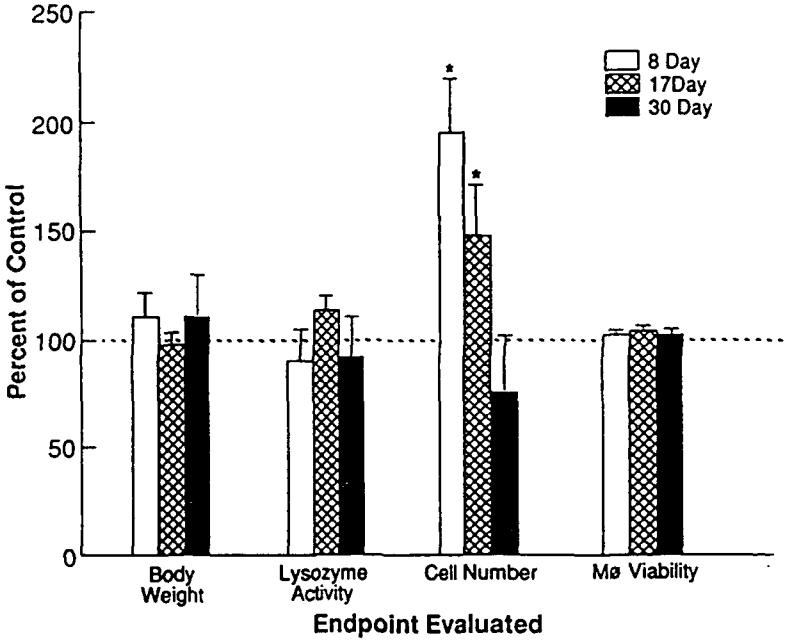

Figure 2. Effects of waterbath exposure to Cd at 2ppb on rainbow trout body weight, lavaged cell number, lysozyme activity in the serum, and macrophage viability (as measured by trypan blue exclusion). While no effects on fish body weight, lysozyme activity, or macrophage viability was observed under the present exposure conditions, cell numbers significantly increased (*p <0.05) after 8 and 17 days of Cd exposure, but declined to control levels after 30 days.

bacterial uptake by their liver and spleen phagocytes, but significantly decreased the rates of intracellular bacterial killing within these cells.

In the studies performed in our laboratory, rainbow trout were exposed to 2ppb Cd in their water (under static conditions) for either 1, 8, or 17 days. The effects on macrophage immune functions essential for maintaining host immunocompetence were then examined.

Macrophages generally exist in the tissues and body cavities as a quiescent population of cells called "resident" macrophages (Zelikoff and Enane, 1991). Upon appropriate stimulation (e.g., bacterial infections), macrophages can become "activated". These cells exhibit a number of morphological, functional, and biochemical differences from the resident cells (Adams and Hamilton, 1984; Enane *et al.* 1993). Since many feral fish are co-exposed simultaneously to environmental contaminants and foreign antigens, effects of Cd were examined in our studies using "activated" trout peritoneal macrophages collected from fish injected IP seven days prior to sacrifice with formalin-killed *A. salmonicida.*

While body burdens of Cd in the gills and liver were elevated in these studies, compared to the Cd levels measured in unexposed control fish (Figure 1), no effects upon body weight, lysozyme activity in the serum, or macrophage viability were observed (Figure 2). However, the total cell numbers recovered in the lavage fluid increased significantly after 8 days of Cd exposure, but then declined with increasing exposure time until baseline control levels were reached after 30 days. The observed changes in lavageable cell numbers appeared to correlate with changes in neutrophil counts (numbers increased at 8 and 17 days and then decreased at 30 days), suggesting that waterbath exposure of rainbow trout to low levels of Cd produced a transient inflammatory response.

In these same studies, *in vivo* exposure to Cd for 30 days enhanced the phagocytic uptake of opsonized latex particles by the trout peritoneal macrophages (Figure 3); a similar response was observed following exposure of trout cells to Cd *in vitro* (Enane *et al.* 1991; Enane, 1991). Exposure to low doses of Cd also produced similar effects on phagocytosis in rodent macrophages (Greenspan and Morrow, 1984). In support of these findings, Robohm and Nitkowski (1976) observed that cunners exposed to Cd in the water for four days had increased rates of bacterial uptake by their liver and splenic phagocytes. The significance of this increased phagocytic activity with respect to overall fish health requires further study.

In addition to the effects observed on macrophage phagocytosis, waterbath exposure of trout to Cd at 2 ppb depressed phorbol myristate acetate (PMA; 1 µg/mL)-stimulated production of H_2O_2 and superoxide (O_2-) (60 min after *in vitro* PMA exposure) by *A. salmonicida*-activated trout peritoneal macrophages 30 days after the initiation of Cd dosing; *in vivo* Cd exposure of mice has also been shown to depress the respiratory burst of pulmonary and peritoneal macrophages (Loose *et al.* 1977). While the results in fish appear contradictory to those observed by Enane *et al.* (1991) following *in vitro* Cd exposure of trout macrophages, it should be remembered that the immunotoxic effects of metals in fish as in mammals is dependent upon a number of factors, including host species, exposure route, and dose and duration of exposure (Treagan, 1975). Reduction in the levels of these cytotoxic mediators (i.e., H_2O_2 and O_2-), factors essential for the aerobic microbicidal defense of many fish species (Nagelkerke *et al.* 1990), may help explain the results of Robohm and Nitkowki (1976) who report decreased rates of bacterial killing by liver and splenic macrophages following exposure of cunners to Cd at 12 ppm. The observed depression in respiratory burst may be due to the inhibitory effects of Cd on cellular respiration (Mustafa and Cross, 1971).

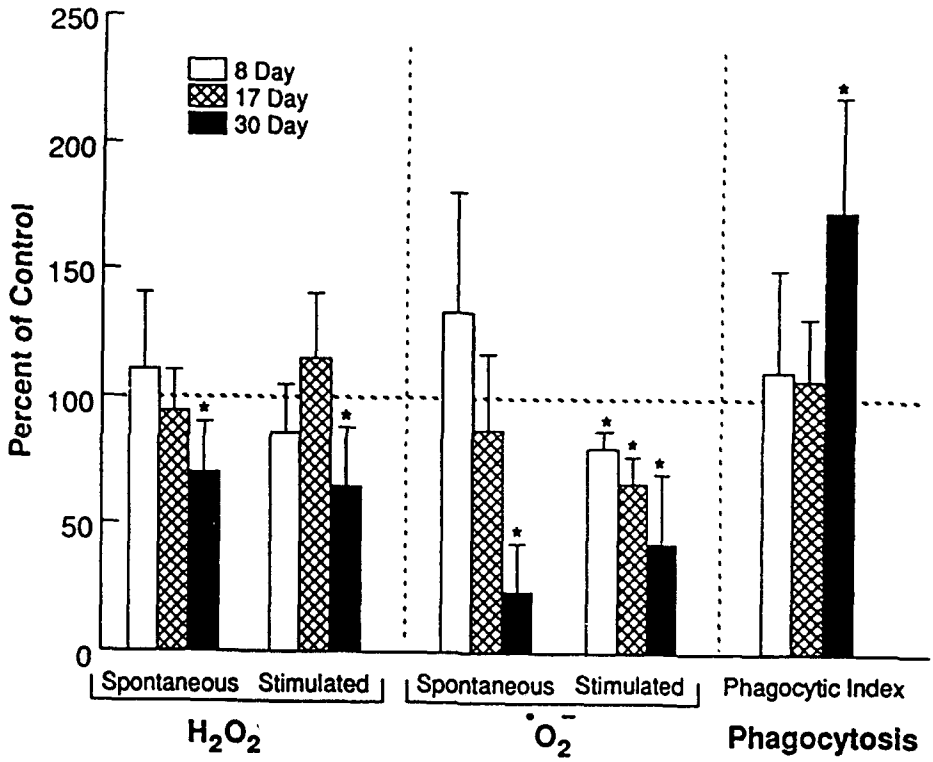

Figure 3. Effects of waterbath exposure to cadmium at 2 ppb on *Aeromonas salmonicida*-activated trout peritoneal macrophage functional activities. Phagocytic index (PI) [(the percentage of macrophage containing trout serum-opsonized latex particles/total number of cells counted) X 100] was significantly enhanced (*p <0.05) after 30 days of Cd exposure. Hydrogen peroxide (H_2O_2) and superoxide (O_2^-) production were significantly depressed with respect to the controls (*p <0.05) by exposure to Cd for 30 days; stimulated production of O_2^- was also significantly depressed (*p <0.05) after 8 and 17 days of exposure. Values represent the mean ±SE of 7 fish.

PMA-stimulated O_2^- production by *A. salmonicida*-activated trout macrophages was reduced as early as 8 days after exposure and the production continued to decline with continuous exposure. These findings support the usefulness of this particular immunological endpoint as a predictor of the immunotoxic effects of environmental Cd exposure, as well as an indicator of the duration of Cd exposure.

In general, the findings from our laboratory studies suggest that macrophage functions, such as phagocytosis and free radical production, may serve as useful indicators to predict the biological effects of low level Cd exposure in feral fish populations. The data also suggest that laboratory studies may help define potential health risks associated with exposure of fish and higher vertebrates to heavily metal-polluted aquatic environments, as well as to provide support for immunosuppression as a link in the etiology of pollutant-associated diseases among marine and estuarine fishes in Cd-contaminated areas.

SUMMARY

Aquatic metal pollution is extensive, and the biological effects on species residing in these waters can be devastating. Organisms directly exposed to these pollutants demonstrate, among other things, an increased incidence of neoplasms and alterations in immunological competence.

Cadmium is a major aquatic pollutant that alters the immune defense mechanisms of fish. In our studies, we have demonstrated that waterbath exposure of trout to a Cd concentration which has no overall effects on fish body weight or on macrophage viability interfered (in a time-dependent manner) with macrophage-mediated activities essential for maintaining host resistance to infectious diseases. Since healthy cellular and humoral immune responses in fish are necessary for protection against diseases, stressors such as Cd could contribute to alterations in the susceptibility of fish to diseases caused by bacterial, fungal, and/or viral pathogens. In order to adequately determine potential health risks associated with Cd exposure on aquatic species, more studies concerning the effects of this toxic metal agent, individually and as a pollutant mixture, are needed.

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Chapter 10

Modulation of Leukocyte Activity by Environmental Chemicals and Parasites in the Eastern Oyster, *Crassostrea virginica*

Robert S. Anderson

Chesapeake Biological Laboratory
University of Maryland
P.O. Box 38
Solomons, Maryland 20688, U.S.A.

ABSTRACT

Circulating hemocytes of the Eastern oyster, *Crassostrea virginica*, can be stimulated to produce cytotoxic reactive oxygen intermediates (ROIs). This event is triggered by phagocytosis or other forms of membrane perturbation, and was quantified by luminol-augmented chemiluminescence (CL) via a liquid scintillation counter modified for single photon monitoring. The respiratory burst so initiated produces ROIs that can participate in antimicrobial defense reactions, or can mediate various manifestations of oxidative stress when produced at levels that exceed the capacity of normal antioxidant defense mechanisms.

The ROI response of oyster hemocytes can be modulated by various endogenous and exogenous factors such as exposure to xenobiotics and/or the presence of intrahemocytic parasites. Exposure of the cells to sublethal concentrations of certain metals (copper or cadmium) or halogenated hydrocarbons (pentachlorophenol) causes a dose-dependent suppression of ROI generation capability. For example, induced CL activity of hemocytes was significantly inhibited (21% inhibited, Bonferroni p value) following 20 hr exposure to 0.5 ppm Cu caused 80% inhibition after 20 hr exposure to 2.0 ppm Cu . Data such as these suggest that CL has potential as a method to screen for potential environmental immunotoxicants. Evidence for the relationship between chemically-induced ROI suppression and immunosuppression in bivalves is found in the increased susceptibility to bacterial infection observed after *in vivo* exposure to the same xenobiotics that produce reduced CL activity *in vitro*. We have also examined the effects of an intrahemocytic parasite (*Perkinsus marinus*) on the CL activity of oyster macrophages. In certain diseases, intracellular parasites either fail to elicit ROI production or actively suppress this activity, thereby helping tc insure their survival. This does not appear to be the case for *P. marinus*; hemocytic CL was shown to be directly correlated with disease intensity, as measured by the numbers of parasites per mL of hemolymph. It was impossible to differentiate lightly infected from uninfected oysters on the basis of CL, but significant differences existed between light, moderate, and heavy infections (Scheffe's S test, p value). It is hypothesized that the presence of the parasites produce a response comparable to macrophage activation and that the excess ROI released by the cells may play a role in the pathogenesis of the disease.

INTRODUCTION

The circulating leukocytes (hemocytes) of bivalve mollusks are of central importance to their defensive capabilities against infectious diseases and parasitism (Cheng, 1981). The hemocytes are macrophage-like cells that rely on lysosomal hydrolases and reactive oxygen intermediates (ROIs) to kill ingested microorganisms. In this discussion, ROI generation by hemocytes will be quantified by luminol-augmented chemiluminescence (CL). As is the case for macrophages from fish and higher vertebrates, it appears that molluscan hemocytes can be activated or suppressed by the presence of xenobiotics or disease.

Mammalian neutrophils and monocyte/macrophages undergo an abrupt increment in oxygen uptake during the processes associated with phagocytosis, followed by the production of ROIs (Badwey and Karnovsky, 1980). The first reaction in this respiratory burst is the one electron reduction of oxygen to the superoxide anion (O_2^-); the reaction is catalyzed by the membrane-associated enzyme NADPH oxidase. Superoxide is converted to hydrogen peroxide (H_2O_2) spontaneously or by superoxide dismutase (SOD), other cytotoxic ROIs such as singlet oxygen and hydroxyl radicals may also be produced by the reduction of oxygen. Hydrogen peroxide in conjunction with myeloperoxidase (MPO) and halide ions forms the basis of a potent antibacterial system (Klebanoff, 1968), the activity of which during phagocytosis is quantified by luminol-augmented chemiluminescence (Stevens and Hong, 1984). Singly or collectively, the ROIs can participate in cell-mediated antimicrobial reactions. The phagocytes and tissues of bivalves and other organisms contain several inducible protective mechanisms against auto-oxidative damage from ROIs such as SOD, catalase, glutathione peroxidase, and vitamins that act as free radical scavengers (DiGuilio *et al.*, 1989; Livingstone *et al.*, 1990). However, when ROI production by hemocytes and by redox cycling of certain pollutants overwhelms the capacity of antioxidant systems, it is likely that pathological consequences of oxidative stress will become manifest.

The production of ROIs by molluscan hemocytes was first reported by Dikkeboom et al. (1985) in snails and by Nakamura *et al.* (1985) in scallops. Work in this area has proliferated since that time; in a recent review of the subject, references were cited on ROI activity in 13 species of gastropods and bivalves (Adema *et al.*, 1991). Many of these studies utilize CL to measure bactericidal activity of the hemocytes, indeed the method has been specifically recommended for use with fish and shellfish (Scott and Klesius, 1981; Wishkovsky, 1988).

Inhibition of macrophage-mediated CL has been used to screen for potential immunotoxic chemicals using cells from laboratory rodents (Tam and Hinsdill, 1990). This approach seems to have similar potential when used with bivalve hemocytes, as described in this paper. Preliminary evidence suggested that hemocyte-mediated antibacterial activity could be compromised in bivalves by exposure to sublethal concentrations of environmental pollutants. For example, in vivo clearance of *Flavobacterium* sp. injected into the hemolymph of *Mercenaria mercenaria* was significantly reduced following exposure to the xenobiotics pentachlorophenol (PCP) or hexachlorobenzene (HCB), and some of the treated clams apparently lost all ability to eliminate the injected bacteria (Anderson *et al.*, 1981). In subsequent in vitro studies of hemolymph withdrawn from PCP- and HCB-exposed clams, it was shown that the bactericidal activity of the circulating hemocytes was impaired (Anderson, 1988). These assays were done by incubating hemocytes with test bacteria, osmotically lysing the hemocytes at various times after phagocytosis, and counting the resultant colony forming units (CFUs). In order to better understand the bactericidal mechanisms suppressed by these experimental procedures, the following studies of chemically induced immunomodulation

were undertaken. Because immunosuppression is generally associated with enhanced susceptibility to disease, ROI production by hemocytes during the course of parasitic infection was also examined.

MATERIALS AND METHODS

Experimental animals

The oysters (*Crassostrea virginica*) were either feral animals collected locally in the Chesapeake Bay (in the case of the pollutant-exposure studies) or hatchery-raised stocks introduced into various locations in the Bay and its tributaries (in the case of the *Perkinsus* infection study). More specific details about the oysters, their sources, their experimental treatments, and the specific methods used can be found in Anderson *et al.* (1992a; 1992b).

Hemocyte-mediated chemiluminescence

The production of ROIs by oyster hemocytes was quantified by measuring luminol-augmented chemiluminescence (CL) before and during phagocytic stimulation. To obtain the hemocytes, several mL of hemolymph were withdrawn from the adductor muscle sinus via a small notch cut in the edge of the valves. The hemolymph was incubated for 15 min at room temperature in a plastic petri dish, during which time the majority of the cells became firmly attached. Filter-sterilized ambient water from the oyster tanks (FA) was used to gently wash off the fluid phase of the hemolymph and any unadhered cells. The cell monolayer was overlaid with FA and incubated for 2.5 hr at room temperature. During this period the cells became progressively more loosely attached and could be finally washed free and resuspended in FA. These cells retained phagocytic activity, but showed little of the clumping typically encountered during the centrifugation and resuspension of freshly collected cells. The hemocytes were removed from suspension by centrifugation (200 g, 15 min), resuspended in cell support medium (CSM), and counted. The composition of CSM was 0.5% antibiotic solution (10,000 U penicillin, 10 mg streptomycin, and 25µg amphotericin B/mL), 5% fetal calf serum, and 1 mg/mL glucose in FA. Six 1 mL aliquots of the hemocyte suspension (10^6 cells in CSM) were placed in small scintillation vials. From this point on, all steps were carried out in a dark room under dim red illumination. To each vial was added 0.3 mL FA and 1 mL luminol (250 µM in FA), and the vials were placed in a scintillation counter programmed for single photon monitoring. The vials were counted at 0.3 min intervals for about 10 min to establish the level of unstimulated, background CL for each sample. Then 0.2 mL of a suspension of heat-killed, washed yeast cells (final particle:hemocyte ratio ≅ 20:1) were added to the vials to provide phagocytic stimulation. The vials were counted at 0.3 min intervals for about 2 hr after the addition of yeast.

Chemiluminescence (counts per min) was recorded directly on computer disks and as printed output from the scintillation counter. The background (basal) CL level was defined as the cpm recorded immediately before the addition of the phagocytic stimulus. The peak CL was the maximal CL response elicited by the phagocytic event (corrected for background). The total CL response was expressed as the area under the phagocytically-induced portion of the curve (estimated by a polygon summation method). Raw CL data were not normally distributed and were transformed (natural log) to permit statistical analysis. If the variance among group means was significant (ANOVA p value <0.05), Scheffe's multiple comparisons test was also performed ($\alpha=0.05$). The computer software used for stastical analyses was Super ANOVA (1989).

In vitro exposure of hemocytes to xenobiotics

The hemocytes were collected and their numbers standardized as described above. The cells in CSM were exposed to sublethal concentrations of the chemical under study for 20-hr intervals in the dark at ≅20°C. The cells were washed and resuspended in fresh CSM, an aliquot was taken from each vial for viability determination by trypan blue exclusion. The CL response of the exposed cells was determined as previously described, and was expressed as percentage of the CL activity of untreated control hemocytes.

CL responses of hemocytes from *Perkinsus*-infected oysters

Uninfected oysters were transferred to a high prevalence *Perkinsus* site on several occasions, following a protocol designed to make available oysters with a wide spectrum of disease stages. The CL response of hemocytes from individual oysters was determined as described above; the activity was measured immediately after harvesting the cells from the plates. The levels of *Perkinsus* infection in each oyster were determined by the methods of Ray (1952; 1966) or Gauthier and Fisher (1990) and the stages assigned numeric values. In this way correlations could be made between stage of infection and CL response activity of hemocytes from individual oysters.

RESULTS AND DISCUSSION

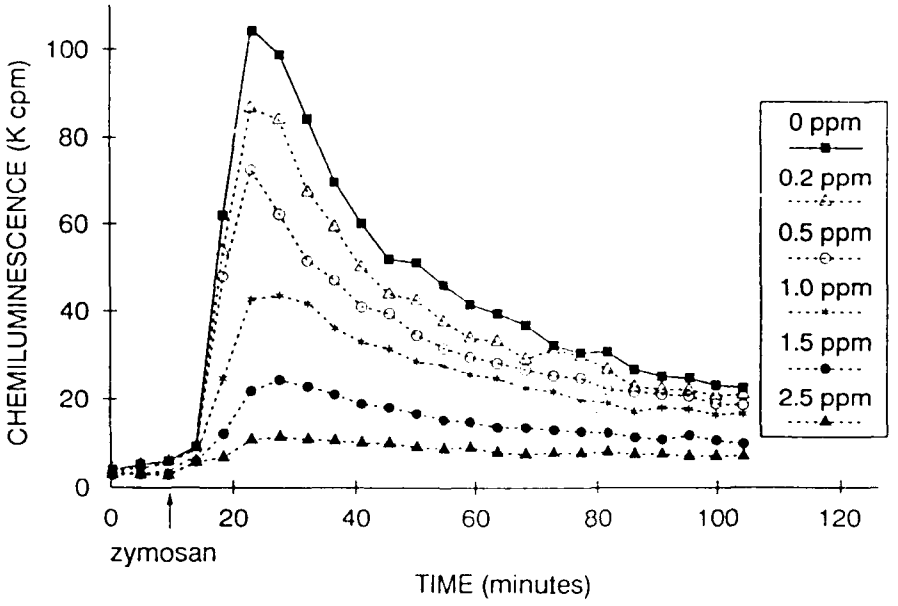

Figure 1. Inhibition of luminol-augmented chemiluminescense (CL) response of *Crassostrea virginica* hemocytes by copper. Control and copper-exposed hemocytes are equal aliquots ($2x10^6$ cells) of the same cell pool, incubated with various copper concentrations for 20 hr at 20°C (room temperature). Resting CL was recorded for 10 min, at which time the hemocytes were phagocytically stimulated by the addition of zymosan particles (20/cell). Chemiluminescent units on the graph x 1,000 = actual recorded counts/min (K).

Table 1
Inhibition of oyster hemocyte chemiluminescence (CL) by 20 hr exposure to Copper sulfate.

Chemiluminescence	Cu (ppm)					Significance
	0.2	0.5	1.0	1.5	2.5	(ANOVA)
Basal CL	10.3 ± 3.9[a]	13.9 ± 4.5	23.1 ± 7.5	30.1 ± 7.4	30.2 ± 7.3	0.0018
Peak CL	9.8 ± 2.5	20.8 ± 5.4	48.8± 7.4	71.1 ± 5.4	82.8 ±4.5	<0.0001
Total CL	8.3 ± 2.7	16.5 ± 5.0	40.3 ± 7.9	65.8 ± 6.2	77.9 ±5.3	<0.0001

[a]mean percent inhibition of control CL ±SEM; N=11 for all exposure groups, except N=9 for 2.5 ppm Cu.

Modulation of ROI production by exposure to environmental toxicants

Hemocytes from several oysters were pooled to obtain ≅1.5 x 10^7 cells for harvesting (as previously described) and six aliquots of 2 x 10^6 cells each were exposed to copper sulfate concentrations ranging from 0-2.5 ppm for 20 hr. The resultant dose-dependent inhibition of hemocyte CL activity of aliquots from a single pool is presented in Figure 1. Even at the higher Cu concentrations tested, hemocyte viability after exposure was ≥ 80%; however, the CL responses at all concentrations were corrected for induced lethality. Cumulative data from 9-11 different cell pools are presented as % inhibition of control values in Table 1. Both basal (background) and phagocytically-induced (peak and total) CL parameters showed the inhibitory effect of Cu, and inhibition of the induced responses was very strong. In Figure 2, the percent inhibition of each of the CL parameters was compared to the control (zero inhibition by definition). The basal CL activity was only inhibited by Cu≥1.5 ppm (Bonferroni-corrected $p < 0.05$), whereas the peak and total CL were significantly inhibited by Cu≥1.0 ppm (Bonferroni-corrected $p<0.001$).

Larson *et al.*, (1989) also studied the CL response of *C. virginica* hemocytes exposed to selected metals or organic compounds *in vitro*, or collected from oysters after various periods of *in vivo* exposure. They reported that copper was the most effective CL inhibitory agent tested, although most of the compounds were CL inhibitory at high exposure levels. Dose-dependent CL inhibition was reported following *in vitro* exposure of oyster hemocytes to 40-400 ppb tributyltin (Fisher *et al.*, 1990). Cadmium has been shown to exert a similar suppressive effect on oyster hemocyte CL (Anderson *et al.*, 1992a). Peak and total CL were significantly reduced by 10 and 2 ppm Cd, respectively, but unstimulated background CL activity was uneffected by all Cd concentrations tested (≤50 ppm). The availability of Cd to the hemocytes was influenced by the composition of the medium, particularly the presence of oyster hemolymph which contained metal-binding proteins. Regardless of the nominal cadmium concentration in the medium, the actual CL inhibition produced was shown to be a function of the intrahemocytic Cd concentration. Similar dose-dependent, chemically-induced inhibition of luminol-augmented CL responses of oysters hemocytes have also been produced by *in vitro* exposure to particulate brass and pentachlorophenol (Anderson *et al.*, 1992c; Roszell and Anderson, 1992). Taken together, these data implicate a number of environmental xenobiotics as potential immunotoxicants because of their inhibitory effects on ROI responses in bivalve hemocytes. However, these are indirect indications of immunosuppression, future work should concentrate on quantifying the effects of these agents on resistance to infectious disease.

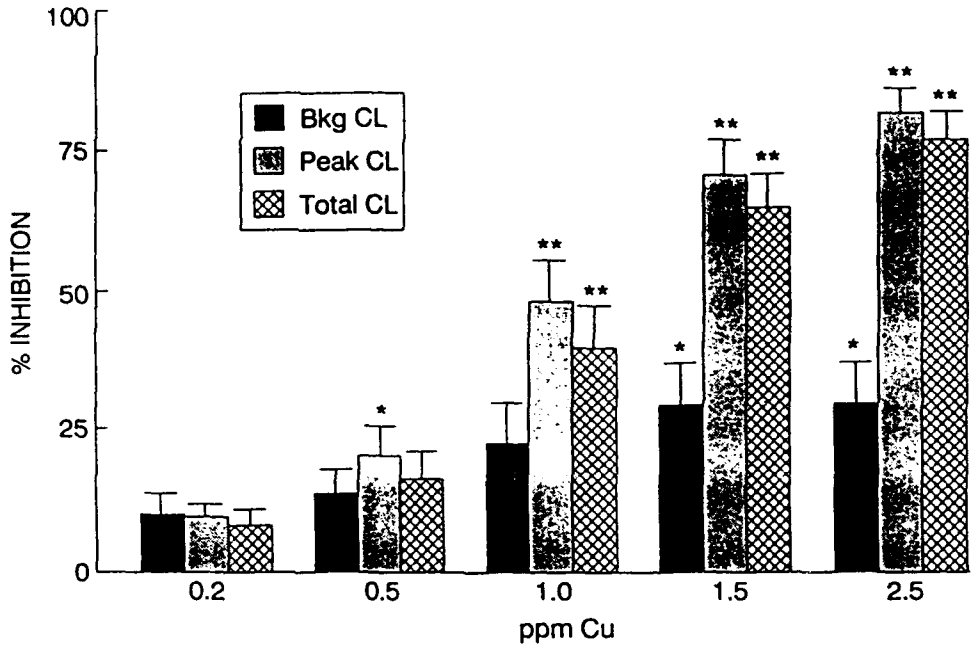

Figure 2. Collected data from 9-11 pools such as that shown in Table 1. Percent inhibition (mean SEM) of hemocyte chemiluminescence (CL) is shown as a function of *in vitro* copper exposure. Effects of Cu on CL of resting hemocytes (Bkg) and on stimulated CL parameters (Peak and Total) are shown; values significantly inhibited as compared to the corresponding unexposed controls are indicated (*$p < 0.05$, **$p < 0.001$; Bonferroni-corrected p values).

ROI production by hemocytes from *Perkinsus*-infected oysters

Perkinsus marinus is a protozoan parasite of *C. virginica* that, along with *Haplosporidium nelsoni*, has decimated the oyster fishery in Chesapeake Bay. Since ROI production (CL) by blood cells is correlated with antimicrobial activity (Welsh, 1980; Horan *et al.*, 1982), our initial hypothesis was that CL responsiveness would decrease as the disease progressed. This impairment of the hemocytes' defensive capacity might explain the intracellular survival of the parasites within the blood cells. In fact, *Bonamia ostreae* an intrahemocytic parasite of the oyster *Ostrea edulis* is phagocytized but elicits no CL response from the host cells (Boulo *et al.*, 1991), although a strong CL response was triggered by ingestion of zymosan (Bachère *et al.*, 1991). A similar phenomenon was reported for Rickettsiales-like organisms that infect the gill tissues and hemolymph of the scallop *Pecten maximus* (LeGall *et al.*, 1989; 1991). However, we found (Anderson *et al.*, 1992b) that hemocytes from *Perkinsus*-infected oysters appeared to be activated, at least by the criterion of enhanced luminol-augmented CL responses to phagocytic stimulation by zymosan.

Both unstimulated and induced CL parameters were measured in hemocytes from individual oysters staged for level of infection, according to the diagnostic index: 0=0; 1=1-10K; 3=10K-100K; and 5≥100K *P. marinus*/mL hemolymph Both the induced CL parameters showed significant graded elevations at all levels of infection. Total hemocyte-mediated CL, stimulated by zymosan ingestion, as a function of disease intensity is given in Table 2. Data on peak CL are not presented because they followed the same trends as total CL activity. The mean total CL responses at the various levels of *Perkinsus* infection differed from each other ($p<0.001$ ANOVA). Post hoc analysis (Scheffe's test, at $\alpha = 0.05$) showed no significant difference between the induced (total CL) response of hemocytes from uninfected and lightly infected oysters, but significant differences exist between all of the other groups as shown in Table 2 (uninfected <moderate < heavy infections). Thus, hemocytes seem to become increasingly responsive to a standard phagocytic stimulus

Table 2
Hemocytic chemiluminescence as a function of disease intensity

Diagnostic index	Total chemiluminescence [area under CL curve, mean ± SD (n)]	Significance of Differences among the means
0 (uninfected)	9,942 ± 7,322 (135)	a
1 (light)	8,907 ± 6,990 (58)	a
3 (moderate)	22,265 ±11,610 (13)	b
5 (heavy)	59,570 ± 37,602 (18)	c

Significant differences shown by different letters; Scheffe's Test, α set at 0.05.

(zymosan) as the disease progresses. We have speculated that increased per cell capacity for stimulated ROI release, perhaps resulting from hemocyte activation associated with the intracellular presence of *Perkinsus* organisms, could mediate oxidant stress-related inflammatory damage that might play a role in the pathogenesis of the disease (Anderson *et al.*, 1992b). The physiological significance of these data generated with zymosan (a yeast cell wall preparation) remains to be fully understood. It should be noted that uptake of *Perkinsus* cells does not seem to elicit luminol-augmented CL by oyster hemocytes (LaPeyre *et al.*, 1992); however, the responses of cells withdrawn from oysters with high intensities of *Perkinsus* infection to ingestion of additional parasites has not been specifically addressed. Furthermore, it may be that hemocyte-produced ROIs are ineffective in protecting the hemocytes. It would be interesting to determine if *Perkinsus* cells produce excretory-secretory products that modulate the oyster's defense mechanisms and interfere with hemocyte killing mechanisms similar to those released by *Schistosoma mansoni* against its snail host (Lodes and Yoshino, 1989; Connors and Yoshino, 1990; Connors *et al.*, 1991).

A conceptual representation of ROI (CL) modulation by chemicals and disease is given in Figure 3. The picture is more complicated than anticipated, but is not unlike that which is known in fish and mammalian species. It is helpful to bear in mind the contrasting roles of ROIs in nonspecific immunity (antimicrobial and tumoricidal properties) vs. their ability to produce local cytotoxic responses and other manifestations of oxidative stress. Not only can certain environmental contaminants cause suppression of CL, but also a slight induction can be observed at very low doses. Dose-dependent inhibition of hemocytic CL is often interpreted as a measure of immunosuppression. Intracellular parasites can produce suppression or induction of ROI release, the exact physiological significance of these phenomena is not clear. The production of extracellular ROIs by activated hemocytes may significantly enhance the oxidant stress already produced in bivalves living in polluted sites via the redox cycling of xenobiotics. Many details need to be worked out, but CL seems to represent a rapid and quantitative measure of the ROI-generating activities of hemocytes from oysters. Some examples have been given in this paper of how CL can be used as a simple screening method to identify potential environmental immunotoxicants and as a short-term *in vitro* test for the analysis of aspects of the host-parasite interaction.

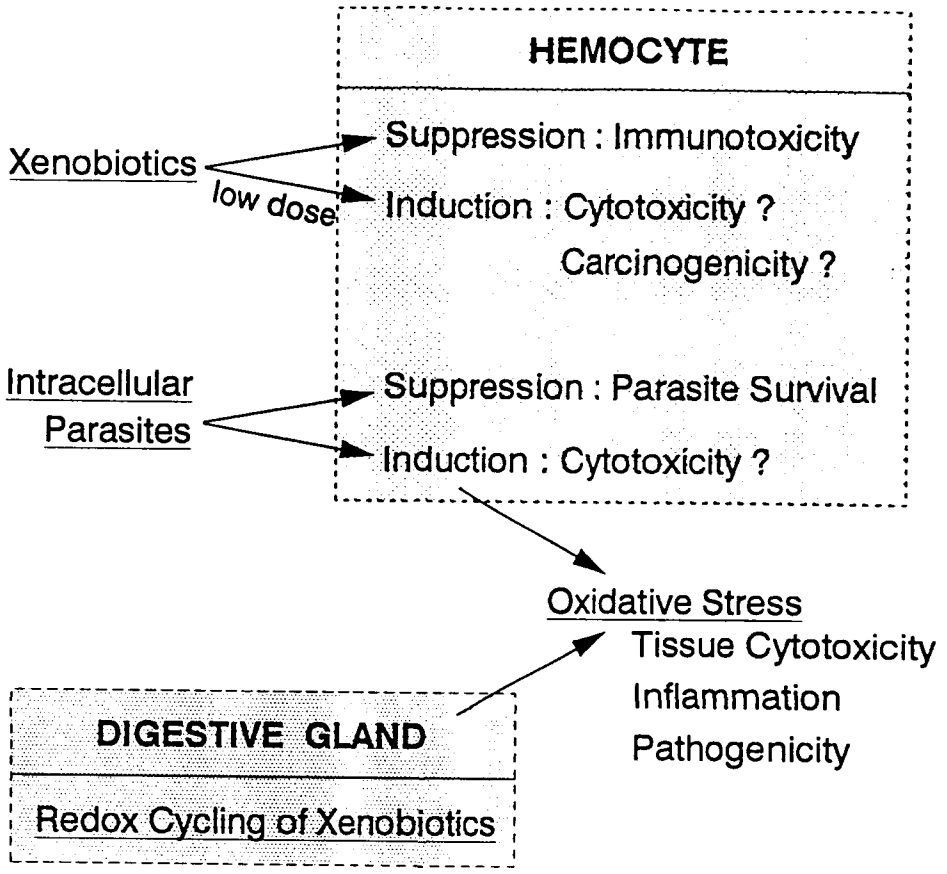

Figure 3. Conceptual representation of the modulation of reactive oxygen intermediates (ROIs) by environmental chemical contaminants and intracellular parasites. Over production of oxygen-derived free radicals in the hemocytes and/or digestive gland could cause intracellular organelle damage by hydrolases released by lysosomal membrane destabilization, or oxyradicals could be released into the extracellular compartment to contribute to pathology associated with oxidative stress. Under production of ROIs could result in reduced protective, antimicrobial capacity of the hemocytes, leading to reduced resistance to parasitic and bacterial infections.

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Chapter 11

Effects of Environmental Contaminants (Pesticides and Metal Ions) on Fish Immune Systems

Muriel B. Dunier

Laboratoire de Toxicologie Métabolique et d'Ecotoxicologie, INRA–Ecole Nationale Vétérinaire de Lyon, B.P. 83
69280 Marcy L'Etoile, FRANCE.

ABSTRACT

Pollutants that originate from agriculture, such as pesticides (insecticides, herbicides, fungicides) and heavy metals or metal ions from industrial wastes are sometimes found at high concentration in the freshwater ecosystem. Most of the xenobiotics are potential immunomodulators and could impair fish health if they alter immunocompetence.

This chapter will review the effects observed on the humoral, cellular, or non-specific immunity of freshwater fish, particularly rainbow trout and carp.

While only impairment of lymphoid organ function was observed in the past, techniques that are currently used allow for the measurement of antibody production (agglutination, ELISA), of antibody secreting cells (ELISPOT), proliferation of lymphocytes with mitogens, graft rejection, cytofluorimetric analysis and various experimental approaches to study phagocytosis.

INTRODUCTION

The influence of some major environmental pollutants (pesticides and metal ions) on the immune system of fish has been reviewed in this paper and shows that chemical pollutants result in a variety of immunomodulatory effects on both humoral and cellular immune functions with a consequence on the health and resistance of fish to diseases. Chronic exposure of fish to xenobiotics has been shown to impair the immune system, as studied in the past (Sindermann,1979, Sinderman *et al.*, 1982; Zeeman and Brindley,1981).

The integrity of the immune system is fundamental for a good defense against a variety of pathogenic agents in the environment, *i.e.*, parasites, bacteria and viruses. The major immune cells, lymphocytes, neutrophils and macrophages, are regulated by a variety of multistep control processes of cellular cooperation and interactions. Macrophages and neutrophils are important factors of the cellular immune system of fish, since they are the first line of defense and function to protect the host by phagocytosing foreign material including pathogenic agents (Ellis *et al.*, 1981).

A good evaluation of the immune response requires a challenge with a pathogen, which usually leads to a rapid lymphocyte proliferation. Rapidly dividing lymphocytes may be more sensitive than somatic ones to effects of xenobiotics and therefore the corresponding immune function.

The complexity and multiplicity of immune responses often make a single test inadequate for evaluating responses of the immune system and thus a battery of tests in most cases is recommended. Furthermore different populations of immune cells may have a variable sensitivity to chemicals. Fish immunotoxicology is a recent, but growing area of research because of increasing interest in the effects of pollutants. Fish are a major sentinel group in the freshwater ecosystem and these lower vertebrates are a good indicator of environmental contamination of water because they can be chronically exposed to highly stable compounds present in water or *via* the food chain (bioaccumulation along the chain), resulting in high levels in predator fish.

IMMUNOTOXIC EFFECT OF PESTICIDES

Insecticides.

Organochlorine insecticides.

The use of **DDT** is now forbidden in Europe and the U.S.A., but African countries continue to use it to prevent diseases where insects are involved. Because it has been extensively used in the past, data are available on its effects on fish immunity. Goldfish (*Carassius auratus*) were injected with various concentrations of DDT and a good correlation was found between dose and decreased levels of total plasma proteins. Furthermore, a leukopenia and a reduction in spleen weight, as well as a suppression of the humoral response to bovine serum albumen was also observed (Zeeman and Brindley, 1981).

Endosulfan is used on seeds. Twenty-five day exposure of salmonids to 0.36 ppb endosulfan was followed by an atrophy of lymphoid organs and a lymphopenia (Zeeman and Brindley, 1981).

Endrin can no longer be used in European countries. A leuco- and a thrombopenia were reported after an endrin exposure in different species (Zeeman and Brindley, 1981). Exposure of trout to sub-lethal concentrations had no effect upon phagocytosis whilst reducing a number of lymphocyte responses (Bennett and Wolke, 1987).

Mirex: A leuco- and a thrombopenia were also reported after mirex exposure (21 or 42 mg/kg/day) in Channel catfish (Zeeman and Brindley, 1981). This hexachloropentadiene is no longer sold in France.

Lindane, the gamma isomer of hexachlorocyclohexane, is one of the most widely used organochlorine insecticides for agricultural purposes, although recently forbidden in France. It is liposoluble and can bioaccumulate in fat. Gluth and Hanke (1985) found that carp may survive only 4 weeks in water containing 100 µg of lindane/ L.The effects of a 3 to 4 month exposure to lindane, given daily in food, on carp humoral response against *Yersinia ruckeri* were investigated by Cossarini–Dunier *et al.*(1987). In spite of significant levels of contamination in lymphoid organs (as measured by gas chromatography) lindane failed to have any immunosuppressive effect on either anti-*Y. ruckeri* antibody production or on spleen weight.The effect of a *per os* exposure (10 mg

lindane/kg carp/day) for 30 day on skin graft rejection was also analyzed (Cossarini–Dunier, 1987). For the first set of grafts, the survival endpoint of allograft rejection was 8 days for lindane-exposed fish as well as for the control fish. For the second set of grafts (mobilization of the immunological memory) it was reduced to 5 days but here too no difference was observed between the two groups. Thus, under these experimental conditions lindane-exposed carp did not delay rejection of either first or second set skin grafts.

The chemiluminescent response of pronephric and spleenic macrophages was analysed after a 2 hr incubation with various concentrations of lindane before opsonized (with normal serum) *Y. ruckeri* were added. The kinetics of production of bactericidal reactive oxygen intermediates showed no difference from controls at lindane concentrations of 10 mg/L and lower (Cossarini–Dunier,1987).

Lindane (10 mg/L) was tested on the *in vitro* replication of the virus of Spring Viremia of Carp in Epithelioma Papulosum Cyprini (EPC) cell lines over 72 hr, no effect on viral replication nor on the cytotoxic effect on EPC cells was observed (Cossarini–Dunier and Hattenberger, 1988).

Table 1 summarizes these data. No effect of lindane was ever observed *in vivo* or *in vitro* on the immune response of carp under these experimental conditions.

Table 1.
Summary of the Effects of Pesticides on Carp Immune Responses

Exposure		Herbicide	Insecticide		
Immune Response		Atrazine	Lindane	Trichlorfon	Dichlorvos
In Vivo	Humoral Response	NE	NE	NE	NE
	Nonspecific Response	ND	ND	↘	↘
	Graft Rejection	ND	NE	ND	ND
in Vitro	Phagocytosis	NE	NE	ND	ND
	Lymphocyte Proliferation	ND	ND	↘	↘
	Viral Replication	NE	NE	ND	ND

ND: Experiment not done, NE: No effect, ↘ = Depression of response

References: Cossari-Dunier (1987), Cossari-Dunier *et al.*, (1987, 1988, 1990, 1991)

Studies on the effects of lindane *per os* (1 mg/kg/ day) or injected (10, 50 or 100 mg/kg body weight) into rainbow trout (*Oncorhynchus mykiss*) showed especially in the second case, a strong depression of the chemiluminescent response of head kidney macrophages and a decrease in lympho-proliferation in response to B mitogens, although the number of B lymphocytes was not modified according to cytofluorometric analyses (Dunier *et al.*, 1993). Some parameters of non-specific immunity, such as serum ceruloplasmin (acute phase protein) were increased while the lysozyme level was decreased only in the trout exposed to lindane *via* injection (Dunier *et al.*, 1993). Antibody secreting cells were slightly affected by exposure to 10 mg/kg lindane and more markedly by 50 and 100 mg/kg. Consequently antibody production was also seriously decreased (Dunier and Siwicki, 1993a).

Catfish exposed to lindane (1.3 mg/L) showed a significant reduction in the total leukocyte count and in the antibody titer against *Aeromonas hydrophila* (Saxena *et al.*, 1992).

Miscellaneous: A continued elevation of serum cortisol (the stress marker) is often associated with impaired immune functions (Fomar, 1993). Aldrin (100 µg/L, 72 hr), dieldrin (20 µg/L, 24 hr), endrin (15 µg/L, 30 day), HCB (100 µg/L, 72 hr), DDT (50 µg/L, 72 hr), DDD (50 µg/L, 164 hr) were reported by Folmar (1993) to increase the serum cortisol in pinfish.

Organophosphorous insecticides

Trichlorfon and Dichlovos: Two major organophosphorous insecticides trichlorfon and dichlorvos are frequently used in pond fish culture in Eastern Europe and therefore may contribute to the pollution of natural waters. Trichlorfon is normally used on cereals, fruit, vine, potatoes, grass and tobacco and dichlorvos (DDVP) is often employed in market garden products, fruit trees and vines. These two highly toxic organophosphorous compounds gain access to freshwater following run–off from fields. Both are inhibitors of cholinesterase, leading to an accumulation of acetylcholine. Trichlorfon and dichlorvos are also used in fish farming for controlling planktonic invertebrates and for treatment and prevention of ectoparasite infections.

According to Woynarovich and Horwath (1980), 0.5 mg/L trichlorfon is used for parasite prevention in nursery ponds. Bathing fish in 0.5 mg/L kills the crustaceans *Cladocera* and *Clopepoda*, aquatic insects, monogenetic trematodes: *Gyrodactylus* and *Dactylogyrus*, *Hirudinea* and parasitic copepods (Jeney and Jeney, 1986). Dichlorvos is commonly used in Scandinavian countries in marine fish farms in the rearing of salmon. The LC_{50} of dichlorvos in rainbow trout is 0.7 mg/L for a 48 hr exposure.

The immunological effects of trichlorfon on carp were investigated by Siwicki *et al.*(1990). In adult carp, the LC_{50} of trichlorfon at 96 hr is 27.5 mg/L and 100 mg/L for young fish (Hoffman and Meyer, 1974).

Trichlorfon effects were studied on several parameters of nonspecific and specific immunity i.e. phagocytic ability and phagocytic index of neutrophils, lysozyme and ceruloplasmin activity in serum and antibody production. Carp were bathed in a medium concentration (10 ppm) or in a very high concentration (20,000 ppm) of trichlorofon. The antibody production against *Y. ruckeri* was not affected (Cossarini–Dunier *et al.*, 1990). Meanwhile, concentrations of 10,000 ppm and 20,000 ppm depressed the nonspecific immune response of carp leading to a leucopenia, a decrease in phagocytic ability of neutrophils, a decrease in lysozyme activity, and an increase in ceruloplasmin activity in serum as observed by Siwicki *et al.*(1990). High doses such as the ones just mentioned are commonly used to spread on ponds in Poland.

A 30 ppm bath exposure of carp for 30 min to dichlorvos, significantly contaminated the spleen, liver and brain as measured by the inhibition of acetylcholinesterase enzymes, but did not affect antibody production (Cossarini–Dunier *et al.*, 1991).

In vitro effects of trichlorfon on the ability of lymphocytes to proliferate in response to T-cell mitogens PHA or Con A, reulted in a strong inhibition by trichlorofon concentrations above 12.5 ppm, a partial inhibition from 10 to 1 ppm, and no effect from 1 ppm to 0.001 ppm.

For dichlorvos, the inhibitory effect occurred at concentrations above 24.5 ppm. For concentrations over 100 ppm trichlorfon and over 24.5 ppm dichlorvos, a total inhibitory effect on head kidney phagocytes (as measured by chemiluminescence) and a dose–related effect for lower concentrations was observed (Cossarini–Dunier *et al.*, 1991). Thus, phagocytic cells as well as the lymphocytes were affected by both organophosphorous insecticides.

Recently, the immunotoxic effects of dichlorvos (2 or 4 mg/L) were tested on sea bass (Obach-Medrano, 1993). It was observed that hematocrits were slightly increased while the number of blood leukocytes was decreased. Serum lysozyme and complement were significantly decreased 2 weeks after immersion in dichlorvos.

Dichlorvos is a well known inhibitor of acetylcholinesterases and of the cholinergic control in release process of lysozyme and other enzymes by neutrophils (Ignarro and George, 1974). The chemiluminescent response of head kidney phagocytes was neither significantly decreased, nor was the antibody response against *Vibrio anguilllarum* (Obach-Medrano, 1993).

Miscellaneous : Malathion (13-178 ppb) had a decreasing effect on spleen weight and on spleen lymphocyte numbers in lake trout and coho salmon (Walsh and Ribelin, 1975).

Methylparathion and **azinphosmethyl** caused a decrease in beta and gamma globulins in the plasma of *Carassius auratus* and *Onchorynchus mykiss*. These compounds have been suspected of modifying the hemopoietic tissue of anterior kidney after a one month bath exposure (Lue-Hung, 1986).

In India, the effects of **organophosphorous insecticides (phorate, phenthoate)** were studied on circulating leukocytes of *Chana punctatus* . The hemoglobin level, as well as the number of small lymphocytes decreased while the large lymphocyte, thrombocyte and total leukocyte counts increased (Chakrabarty and Banerjee, 1988).

Carbamates:Walsh and Ribelin (1975) described an atrophy of lymphoid organs and a depletion of lymphocytes in salmonids after a 100 day exposure to carbaryl at 10.2 ppm. At 0.1 ppm a considerable increase in susceptibility to sporozan parasites in spot (*Leiostomus xanthurus*) was observed (Zeeman and Brindley, 1981).

Herbicides

Atrazine (2–chloro–4–ethylamino–6–isopropylamino–trazine), one of the most widely used herbicides in agriculture is very effective against weeds in many crops, and is often used on apple trees and corn in Europe. It is slightly soluble in water (28 mg/L) and contaminates aquatic environments by run–offs from cultivated fields. In France, 10 % of the pollution leading to death of fish was due to atrazine (Belamie and Giroud, 1986).

Biagianti-Risbourg (1990) studied the effects of sub-lethal concentrations of atrazine on the melano-macrophages centers in the livers of the mugilids *Liza ramada* and *Liza aurata* and observed degenerated macrophages surrounded by monocytes and small lymphocytes. After a 7 day bath exposure to 0.025 to 0.28 mg/L atrazine, a resistance to pollution was observed in the liver while the number and the size of melano-macrophage centers were enhanced. Atrazine in concentrations

of 0.1 to 1 mg/L was reported to produce a leucopenia and an atrophy of lymphoid organs in salmonids (Walsh and Ribelin, 1975).

The consequences of long term, moderate exposure of carp to atrazine *per os* (0.1, 1 or 10 mg/kg body weight/day) were analysed by antibody production against *Y. ruckeri* and by the splenic index. No effect was reported (Cossarini-Dunier *et al.*,1988b). *In vitro* exposure of head kidney macrophages to 7 to 28 mg atrazine/L had no effect on the chemiluminescent response (Cossarini–Dunier, 1987). A concentration of 28 mg atrazine/L *in vitro* during the 72 hr kinetic studies of the replication of Spring Viremia of Carp virus in EPC cell line did not modify the kinetic response(Cossarini–Dunier and Hattenberger, 1988). Thus, *in vitro* or *in vivo* exposure to atrazine under these experimental conditions had no effect on the immune functions of carp or on virus replication.

Recently, **atrazine** (100µg/L, 72 hr) was reported (Folmar, 1993) to increase serum cortisol concentration in pinfish. The stress due to atrazine pollution is thus associated with a possible impairment of immunity.

Other herbicides

2,4 Dichloro–phenoxyacetic acid (2-4 D) in concentrations of 0.35 to 430 ppm, was reported to decrease the spleen weight and the number of spleen lymphocytes in lake trout and coho salmon (Walsh and Ribelin, 1975).

Diuron, 3-(3,4 dichlorophenyl)–1,1–dimethylurea, in concentrations 2.4 to 10.2 ppm in roach and ide was reported to modify the electrophoretic pattern of plasma proteins (Komorovsky, 1972).

Diquat (9,10 dihydro–8a,10a-diazoniaphenanthrene) and **endothal** (7-oxabicyclo (2,2,1) heptane–2,3–dicarboxilic acid) used to control algae and aquatic weeds (0.11 to 23 ppm) decreased lymphocytes and increased thrombocytes in the sera of blue gill and goldfish but had no effect on total plasma proteins or on head kidney hematopoietic tissue (Berry, 1975)

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), a chlorinated dioxin used in the 1960's as a defoliant (orange agent) proved to be hypophagic and to suppress hematopoiesis in rainbow trout injected with 10 µg/kg (Spitsbergen *et al.*,1986, 1988).

Fungicides

Pentachlorophenol (PCP) found widespread applications as a fungicide, an antimicrobial agent, a molluscicide and mostly as a wood preservative. It is a common estuarine and marine pollutant (Schuller *et al.*,1985). The *in vitro* effect of analytical (A) and technical (T) grade PCP on the chemiluminescent response of phagocytic cells from anterior kidney was studied in the Japanese mekata *(Oryzias latipes)*. A and T-PCP both produce a dose-dependent and reversible immunomotoxic effect (Anderson and Brubacher, 1992). Furthermore, after a 20 hr exposure to T-PCP the suppressive effect proved greater than following exposure to A-PCP.

PCP in water (at 1 ppm) was also reported to affect the total plasma protein level in European eel, *Anguilla anguilla* (Holmberg *et al.*, 1972).

HEAVY METALS AND METAL IONS

Because fish are frequently exposed to many industrial pollutants in their aquatic environment, their immune system may also become compromised by metal ions.

Manganese

Manganese (Mn) ion is released into freshwater ecosystems by industries such as steel and mining and those which manufacture paints, textile dyes, ceramics, fertilizers, and fungicides such as maneb. This latter pesticide is usually employed to treat watercress ponds and thus is often implicated in pollution of rivers. Lethal concentrations at 24 hr for fish to $MnCl_2$ and Mn SO_4 are respectively 5.5 and 3.4 g/L (Agrawal and Srivastava, 1980).

Anemia and leukocytopenia were observed by Wepener *et al.*(1992) in tilapia exposed to Mn originating from mining.

The kinetics of anti-*Yersinia ruckeri* antibody response following an exposure for 2.5 months to 50 mg/L $MnCl_2$ in water, was studied and no difference was observed between exposed carp and control fish (Cossarini-Dunier *et al.* , 1988a). Mn concentration in lymphoid organs (spleen and kidney) increased from day 14 to 35 with a concurrent decrease in hematocrit after 35 day. Manganese enhanced the phagocytic ability of spleen and head kidney macrophages for opsonised *Yersinia ruckeri* from 6 to 50 mg/L *in vitro* (Cossarini–Dunier, 1987).

Other studies of cellular immunity in carp have been reported (Ghamni *et al.*, 1987, 1989): Mn at 10^{-1} M inhibited lymphocyte proliferation with Con A and PHA when added *in vitro* 16 hr prior to the beginning of the culture. Although proliferation after LPS stimulation was unaffected, Mn acts as a stimulant from 10^{-7} M to 10^{-3} M.

The natural killer (NK) cytotoxicity of pronephros cells against YAC-1 and P 815 target cells was increased following exposure to Mn at a concentration of 60 µg Mn per culture . When injected *in vivo* 80 µg/g body weight also stimulated the NK function (Ghamni *et al.*, 1990).

Magnesium

Dunier and Siwicki (1993b) studied the *in vitro* effect of $MgCl_2$ on two immune functions of carp. A suppressive effect was observed on lymphocyte proliferation for all concentrations (Table 3). The effect of $MgCl_2$ *in vitro* on the phagocytosis of *Y. ruckeri* in carp was suppressed at high concentrations and a stimulated at very low concentrations (Table 2).

Zinc

Guppies were subjected to 5 ppm $ZnSO_4$ for extended periods (Zeeman and Brindly, 1981). When exposed for 55 to 65 day, there was some reduction of lymphoid tissue in the head kidneys and those exposed for 95 day had reduced spleens weights.

Dunier and Siwicki (1993b) described a dose–dependent immunomodulation of phagocytosis and a total suppression on lymphocyte proliferation with $ZnCl_2$ (Table 2 and 3).

Table 2
Effect of Metal Ions (values in ppm:mg/L) *in Vitro* on spleen and head kidney macrophages of carp; the phagocytosis studied by chemiluminescence*

Metal	Stimulation	Equal to Control	Partial Suppression	Total Suppression
Cu	-	6.2	12.5-50	50-100
Mg	0.01	0.2-3.1	6.2-100	-
Pb	-	1-100	-	-
Zn	50-100	25	3-12	200-400

* Dunier and Siwicki, 1993b

One ppm $ZnSO_4$ for 38 weeks in the water of trout or carp suppressed the antibody response against MS2 bacteriophage, and enhanced the secondary response to the second bacteriophage challenge (O'Neill , 1981a, b).

For 8 weeks, $ZnSO_4$ was added weekly to tanks containing *Brachydanio rerio* and a total inhibition was observed at 70 ppm on humoral response against *Proteus vulgaris* but no effect was observed against infectious pancreatic necrosis (IPN) virus (Sarot and Perlmutter, 1976).

Ghamni *et al.*(1989) incubated carp lymphocytes with 10^{-7} M to 10^{-3} M Zn and they observed an inhibition of the proliferation of lymphocytes stimulated with Con A, PHA and LPS at 10^{-5} M. Stimulation of T and B cells occurred at 10^{-3}M.

Guppies exposed for 95 day to 10 ppm zinc sulfate showed decreased spleen size and an extensive degeneration of many organs (Crandall and Goodnight, 1963). McLeay (1975) observed a leucopenia to acute zinc sulfate concentrations and a decreased resistance of coho salmon to disease.

Table 3
Effect of metal ions (values in ppm:mg/L) *in vitro* on spleen and head kidney lymphocytes of carp stimulated by PHA-P mitogen*

Metal	Stimulating Effect	Equal to Control	Partial Suppression	Total Suppression
Cu	-	-	0.3-5	10-100
Mg	-	-	3-100	-
Pb	6-100	3	-	-
Zn	-	-	-	0.75-200

*Dunier and Siwicki, 1993b

Copper

Copper sulfate ($CuSO_4$), a well known immunotoxic compound in mammals, is used in fish farms for antiparasitic treatment and many studies are available in the literature. Antibody response to MS2 bacteriophage was completely suppressed in carp exposed to 0.29 ppm $CuSO_4$ (38 weeks). At this dose and time, primary blood clearance of live bacteriophages was increased in *Salmo trutta* as well as in *C. carpio*, O'Neill (1981a, b).

Copper chloride ($CuCl_2$) was tested *in vitro* by Dunier and Siwicki (1993b) on carp leukocytes. All concentrations above to 6 mg/L were suppressive to phagocytosis (Table 2). Lymphocyte proliferation was suppressed at doses up to 0.3 mg/L $CuCl_2$ (Table 3).

Elsasser *et al.* (1986) observed a suppressive effect in rainbow trout on the phagocytic uptake of *Staphylococcus aureus* as measured by chemiluminescence : 1 to 10 mg/L was strongly suppressive while 0.1 mg/L and lower was not.

Stevens (1977), observed that juvenile coho salmon exposed for 1 month to 10 to 33 ppb $CuCl_2$ exhibited a decreased humoral response against *Vibrio anguillarum.*

Baker (1969) observed a large reduction and necrosis of the interstitial hematopoietic tissue in the kidneys of the flounders exposed to copper.

Baker *et al.* (1983) found that a sublethal copper exposure would increase the susceptibility to infection of chinook salmon and rainbow trout to *Vibrio anguillarum*. Mortality due to the infection was greatest in fish exposed to *40* µg/L Cu for 96 hr. After the stress due to Cu pollution, rainbow trout required about 50 % fewer pathogens to induce a total infection than in control fish.

Roales and Perlmutter (1977) showed that 9 ppb copper decreased the immune response of the blue gourami (*Trichogaster trichopterus*) to viral (IPN) and bacterial antigens *(Proteus vulgaris*), with an abnormal reduction of hemosiderin bodies in the white pulp of spleen. An increased susceptibility to IHN virus was observed in rainbow trout exposed to sublethal concentrations of copper (Hetrick *et al.*,1979).

Acute exposure to copper (1 to 5 µg/L) modified the susceptibility of steelhead trout to *Yersinia ruckeri* (Knittel, 1981). The lethal concentration of Cu for trout is 10 µg/L for an 8 day exposure.

Juvenile rainbow trout were maintained in copper nitrate (0.05 ppm) for 43 days and an increase of total immunoglobulin was observed with no modification of the proliferative responses to mitogens. A challenge with *Saprolegnia parasitica* induced a significant increase in the cortisol level (Carballo *et al.*,1992). The lack of correlation between the effect of stress and a modulation of the immune response would suggest a direct effect of these toxicants instead of a stress- mediated mechanism.

Immunosuppression was demonstrated by Anderson *et al.* (1989) in sections of rainbow trout spleens immunized *in vitro* with DNP-Ficoll and exposed in culture to copper chloride concentrations of 0.1 to 10 g/mL . After 10 day, the number of antibody-producing cells, as measured by the PFC assay, was reduced.

Japanese eels exposed to copper (100 to 250 µg/L) for 12 hr demonstrated an increased level of corticosteroids and a decrease of about one third of lymphocytes and granulocytes (Mushiake *et al*, 1985); the phagocytic or uptake rate was reduced for *Edwardsiella tarda* but not against *Vibrio anguillarum*.

Lead

The immunotoxic effects of lead on fish was first studied about 60 years ago. Brown Bullhead exposed for 183 day to 50 ppm lead acetate demonstrated a reduction in phagocytic activity by peripheral phagocytes and phagocytes present in lymphoid organs. A reduced hematopoietic activity in spleens was also observed (Dawson, 1935).

More recently, Dunier and Siwicki (1993b) exposed carp cells *in vitro* to $Pb(NO_3)_2$ and observed no effect on phagocytosis but a stimulation of lymphocyte proliferation at lead concentrations over 6 mg/L (Table 2 and 3).

A reduction in the size of the spleen and the head kidney after a 2 month exposure of guppies to 5 ppm lead nitrate was observed by Crandall and Goodnight (1963), as well as a relative increase in the total number of lymphocytic and granulocytic cells in the blood.

Carp exposed for 40 day to 1 ppm lead acetate demonstrated a reduction in serum albumin and gamma–globulin levels in the electrophoretic patterns in sera (Fujiya, 1961).

Salmo trutta injected intraperitoneally with low concentrations of $Pb(NO_3)_2$ exhibited a decrease in antibody titer against MS2 bacteriophage (O'Neill, 1981a).

Mercury

Roales and Perlmutter (1977) showed that 9 ppb methylmercury exposure of 4 to 5 weeks decreased the immune response of the blue gourami to viral (IPN) and bacterial antigens (*Proteus vulgaris*), with damage to the splenic white pulp.

Voccia *et al.* (1994) studied the functional impairment of the major immune cells of rainbow trout by methylmercury and mercuric chloride *in vitro*. The cytotoxic potential of methylmercury appeared to be at least ten-fold greater than that observed for the cytotoxicity of mercuric chloride (10^{-5} M versus 10^{-4} M). Impairment of the functional activities (lymphocyte proliferation by mitogens, mixed leukocyte reaction, phagocytic and respiratory burst activity of blood and head kidney macrophages) appeared to be limited almost exclusively to cytotoxic mercury concentrations. A nonspecific, poisoning-related impairment of immune function was implied from these studies.

Cadmium

Gardner and Yevich (1970) exposed mummichog to cadmium (Cd) for 48 hr and observed a slight decrease of lymphocyte numbers, a marked decrease of thrombocytes in the circulation and a striking increase of head kidney granulocytes, after 8 hr .

A significant reduction of the total leukocyte count and antibody titer against *Aeromonas hydrophila* in catfish exposed to Cd (0.2 mg/L) was found by Saxena *et al.* (1992).

Cd has been demonstrated to affect the immune response of rainbow trout (Viale and Calamari, 1984, Zelikoff, these proceedings). A single dose of Cd injected intraperitonelly to *Salmo trutta* decreased the humoral responses against the MS2 bacteriophage (O'Neill, 1981a).

Paradoxical effects of Cd exposure were observed by Robohm (1986) on *Bacillus cereus* antibody response in two fish species; an inhibition in cunner (*Tautogolabrus adspersus*) at 12 µg/mL and an enhancement in striped bass (*Morone saxatilis*) at 10 µg/mL.

The cellular response of fish previously immunized with *V. anguillarum* to the homologous antigen was significantly lower for splenocytes obtained from fish exposed to 3.6 µg/L Cd for 9 weeks than for controls; the humoral response was higher, even though the leukocyte counts were not modified (Thuvander, 1989).

Cd proved to impair antigen-binding cell functions in the bluegill (*Lepomis macrochirus*) as studied (Kusher and Crim, 1991).

Associations of different compounds

Copper and methylmercury: Roales and Perlmutter (1977) showed that 9 ppb copper and methylmercury applied coincidently decreased immune responses of the blue gourami to viral (IPN) and bacterial antigens (*Proteus vulgaris*), as well as reduced hemosiderin bodies in the white pulp of spleen.

Cadmium and lindane: The peak antibody titer against *Aeromonas hydrophila* in catfish exposed to Cd (0.2 mg/L) and lindane (1.3 mg/L) was found to be significantly suppressed (Saxena *et al.*, 1992). A significant reduction in the total leukocyte count and in hematocrits was also observed.

Methylmercuric chloride and chlorobenzene: Rainbow trout were exposed to methylmercuric chloride (150, 13 and <10 ng/L) and a mixture of 10 chlorobenzene compounds (2,350, 115 and 19 µg/L) in water and the red and white blood cell ratios were examined. Little or no change was observed in these parameters, even when fish were exposed for 75 to 119 day (Niimi and Lowe-Jinde, 1984). The authors concluded that cell ratios are not suitable indicators of contaminant stress in fish.

CONCLUSION

There has been an increased interest in the effect of environmental pollution on fish health and on their immune systems, but the data are still sparce and sometimes contradictory. The complexity of the immune system often makes a battery of tests more suitable than a single test; different cell populations may have a variable sensitivity to chemicals. The variability between the different species of fish and the difficulties in evaluating effects and then in interpreting the mechanisms make fish immunotoxicology a difficult field. We can expect interesting development in immunotoxicity in the near future.

The mechanisms of immunomodulation caused by pollutants could depend on whether there is a direct toxic effect on immune cells and organs, or via an indirect effect, on the neuroendocrine system. Salmonids are particularly sensitive to stress which affects glucocorticoids and can be detected by an increase in the plasma cortisol level, (Kaattari, 1991). The consequence is a selective suppression of essential interleukins, a modification in the sensitivity of lymphoid organs and of the affinity of leukocyte receptors. Kaattari and Tripp (1987) showed that cortisol decreases specific B cell precursor numbers in fish and this could account for a decreased number of specific antibody secreting cells following pollutant exposure. This could explain why stress has been reported to suppress antibody responses and reduce host resistance to disease (Peters *et al*, 1988). A significant decrease in leukocyte counts may be the result of increased secretion of corticosteroids (Ellis, 1981). This secretion is a nonspecific response to any environmental stresssor and is a basic mechanism underlying the increased susceptibility of fish to diseases.

Some products (α and β adrenergic receptor antagonists) of the peripheral sympathetic nervous system of the rainbow trout can modify the antibody response in splenic lymphocytes as well as the respiratory burst of pronephric leukocytes (Flory,1988; Flory and Bayne, 1991).

Histological studies of pronephric tissue showed that under stress, phagocytes may become activated, with an observed increase in the phagocytosis rate. They also showed marked hypertrophy, increased pseudopod formation and enhanced autophagocytosis, however, many pronephric macrophages from stressed trout showed signs of degeneration: necrosis, lysosomes rupture and cytolysis (Peters *et al.*,1991). Stressed fish showed a breakdown of the natural cytotoxicity and a marked lymphocytopenia. Macrophages retained their capacity as presenting cells when exposed to T lymphocytes (Peters *et al.*,1991).

Fish are excellent models to aid in risk assessment for immunotoxicology. Risk assessment is a process whereby relevant biological, dose-response and exposure data for a xenobiotic are analysed in an attempt to establish qualitative and quantitative estimates of adverse outcomes.

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Chapter 12

Modification of Immune Function In Winter Flounder by Cortisol Administration and Putative Contaminant Exposure

R.E. Carlson[1] and J.E. Bodammer[2]

[1] Department of Fisheries, Animal and Veterinary Science
University of Rhode Island
Kingston, RI 02881

[2]URI/NOAA Cooperative Marine Education and Research Program
University of Rhode Island
Kingston, RI 02881

ABSTRACT

Winter flounder (*Pleuronectes americanus*) were sampled from the Niantic River (NR), a non-urban estuary, and from the Morris Cove (MC) area of New Haven Harbor, a contaminated bay. Immunological assessment using the *in vitro* primary plaque-forming cell (PFC) response as measured by the passive hemolytic plaque assay was conducted on fish from both sites. Splenic lymphocyte suspensions from MC flounder had a significantly lower mean PFC response than lymphocytes from NR flounder when cultured for 9 day with trinitrophenyl-lipopolysaccharide (TNP-LPS) or when cultured for 14 day with trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH). There were no significant differences in the mean PFC responses of NR and MC flounder when splenic lymphocytes were cultured for 9 day with TNP-KLH. Additionally, there were no significant differences in the mean serum cortisol and hematocrit of MC and NR flounder. Fish from both sites had no gross external or internal lesions; however, flounder from NR had a significantly higher condition factor than flounder from MC. Our results indicate that the *in vitro* primary PFC response can be used to evaluate the presence of chronic stress in winter flounder and may provide an effective immunological indicator for environmental monitoring.

INTRODUCTION

Immunological assays have been suggested as potential methods for monitoring aquatic environmental degradation (Adams, 1990; Weeks *et al.*, 1992). Immunological assessment of wild fish populations using the passive hemolytic plaque assay (PHPA) may be possible by the removal of lymphatic tissue immediately upon collection from the environment and measurement of the *in vitro* primary immune response. *In vitro* stimulation (immunization) of lymphatic tissue using trinitrophenyl-conjugates has been accomplished in cultured fish species (Miller and Clem, 1984; Kaattari *et al.*, 1986) and eliminated the variability of antigenic exposure between different groups

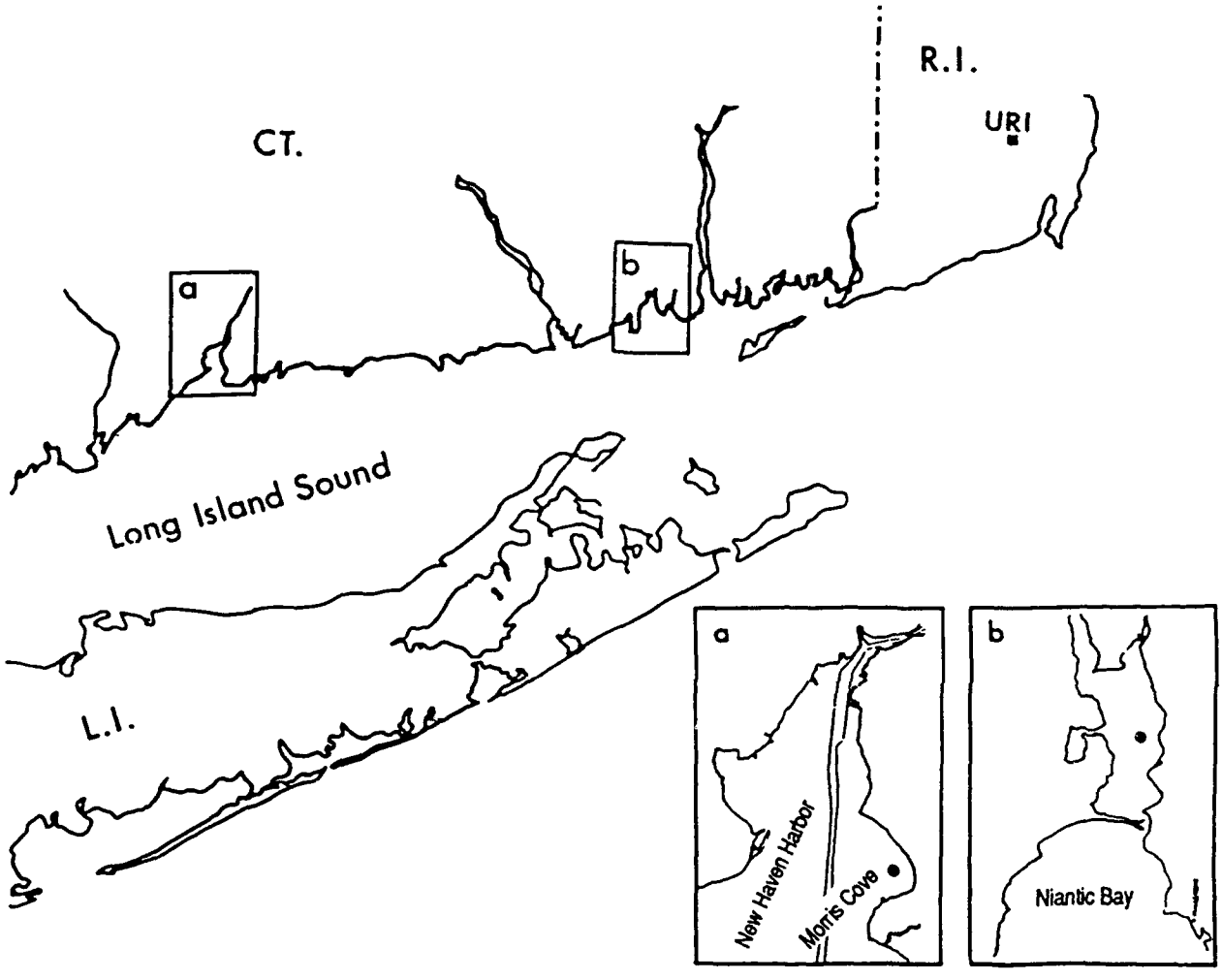

Figure 1. Map of Long Island Sound.. Inserts are enlargements of New Haven Harbor (a) and the Niantic River (b). The dot (●) represents the sampling site in each area.

of fish (Robohm *et al.*, 1979). This technique has recently been used to study the immunosuppressive effects of xenobiotics on the specific immune response of chinook salmon (*Onchorynchus tshawytscha*) (Arkoosh *et al.*, 1991). The tendency of winter flounder (*Pleuronectes americanus* L.) to maintain resident populations in bays and estuaries of the Northwestern Atlantic seaboard (Olla *et al.*, 1969; Crawford and Carey, 1985) makes it an ideal species for environment monitoring (Murchelano and Wolke, 1991). Immunological studies on the specific immune response of captive or feral winter flounder have been limited to the measurement of *in vivo* antibody production (Robohm *et al.*, 1979; Stolen *et al.*, 1984; Laudan *et al.*, 1989). These studies have suggested that the immune system of winter flounder is similar to other flatfish species and may be sensitive to environmental stressors.The effects of heavy metals (Kohler, 1990), endotoxin (White *et al.*, 1984), hypoxia, and confinement (White and Fletcher, 1989) have been studied in other flatfish species such as plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*). This paper presents the application of an *in vitro* primary PFC assay to winter flounder collected from a minimally contaminated and heavily contaminated site. The results will be related to a previous study (Carlson *et al.*, 1993) in which suppression of the *in vitro* primary immune response occurred in groups of captive winter flounder following implantation of a cortisol-containing vehicle.

MATERIALS AND METHODS

Fish

Winter flounder (mean length 33.1 ± S.E. 0.4 cm and weight 514.7 ± 12.7 g) were collected in the Niantic River (NR) and/or Estuary (Long Island Sound, Waterford, CT) (Figure1) by otter trawl (Northeast Utilities Environmental Laboratory,Waterford, CT). Winter Flounder (mean length 31.7 ± 0.9 cm and weight 395.0 ± 12.7 g) were collected in Morris Cove (MC) (New Haven Harbor, Long Island Sound, New Haven, CT) by otter trawl (R/V Shang Wheeler, National Marine Fisheries Service). Fifteen flounder were collected from each site, and collections were done one week apart during the month of May, 1992. Water temperature at the NR site was 13.5°C and salinity was approximately 27.4 ppt. Water temperature at the MC site was 12.0°C and salinity was approximately 23.5 ppt. Fish were collected using 15 to 30 min trawls and were placed into a holding tank on board each vessel until sampled (MC) or transferred to a dockside holding tank prior to sampling (NR). No fish was held longer than 30 min.

Sampling procedure

Each fish was rapidly netted from the holding tank, anesthetized with tricaine methanesulfonate (MS-222) (Argent Chemical Co.,Redmond, WA) at 200 mg/L and weighed. A caudal sinus venipuncture was performed and the fish exsanguinated. Approximately 1 mL of whole blood was placed into a cold vacutainer tube, 1 mL placed into a cold heparinized vacutainer tube, and 80 µl placed into two heparinized microhematocrit tubes. Each fish was then killed by a sharp blow to the head.The skin covering the peritoneal cavity was surgically prepared by scrubbing with 7.5% providone iodine scrub solution (BetadineSurgical scrub, Purdue Frederick Co., Norwalk, CT) followed by wiping with 100% isopropyl alcohol. The right (pigmented) abdominal wall was removed. The spleen was dissected free from mesenteric attachments and placed into a sterile, 40-mL, screw-cap tissue culture flask (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) containing 10 mL of cold (7°C) sterile RPMI 1640 media with L-glutamine (Cellgro, Mediatech, Washington, DC) and 50 µg/mL gentamicin sulfate (Sigma Chemical Co., St. Louis,MO).^ The

spleens were kept cold during transport to the University of Rhode Island, Kingston, RI. Following splenectomy,the length, reproductive status, and presence of gross lesions were recorded.

Cortisol determination.

A validation experiment was performed using winter flounder serum and a solid phase radioimmunoassay system (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) as described by White and Fletcher (1984). Following validation, all flounder serum cortisol measurements were done in duplicate and the average recorded.

Lymphocyte preparation and culture

The contents of each flask was transferred to a 60 x 15 mm sterile plastic Petri dish, and the volume of the media was adjusted (6 mL to 9 mL) according to the spleen size. A 1 mL syringe was used to dissociate tissue. Then, 2 mL of suspension was transferred to each of three wells of a sterile 12-well, flat bottom, cell culture dish (Gibco, Grand Island, NY). Initial culture density was determined using a hemocytometer and ranged from 1.0×10^7 lymphocytes/mL to 5.2×10^7 lymphocytes/mL. The mean culture density was $3.07 \pm 0.3 \times 10^7$ lymphocytes/mL. Initial lymphocyte viability was determined using trypan blue exclusion and exceeded 95% for all suspensions. Autologous plasma (DeKonig and Kaattari, 1991) was heat inactivated (42°C for 40 min) and 100 µl added to each culture. Trinotrophenylyl-lipopolysaccharide (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975) and 10 µl of 0.1 mg/mL of TNP-LPS added to one of the three wells. Trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) was prepared by the technique of Rittenberg and Amkraut (1966) and 10 µL of 1.0 mg/mL TNP-KLH added to the two remaining wells. The lymphocyte-antigen suspensions were placed into an airtight incubator (Model 3-12,C.B.S. Scientific Co., Del Mar, CA) with 5% carbon dioxide in air and incubated at 15°C for 9 day. The suspension containingTNP-LPS and one of the two suspensions containing TNP-KLH from each flounder was removed for use in the PHPA (9-day assay). The remaining suspension containing TNP-KLH was cultured for an additional 5 day before use in the PHPA (14-day assay).

Passive Hemolytic Plaque Assay

Following incubation, a 3 mL syringe was used to aspirate each suspension and expulse it through a disposable filter (60µm, Precision Laboratory Plastics, Bothell, WA) into a 15 mL centrifuge tube containing 5 mL of cold RPMI 1640 media. The tubes were centrifuged at 770 x g for 6 min at 4°C. The supernatant was removed and the cell pellet resuspended in cold RPMI 1640 to an appropriate lymphocyte concentration for the PHPA. Each tube was stoppered following the addition of carbon dioxide (5%) in air and kept on ice.

TNP-labelled SRBC were prepared for use in the PHPA as described by Rittenberg and Pratt (1969) and used as 25% solution. Plaque assays were performed using the method of Cunningham and Szenberg (1968) as modified by Blazer and Wolke (1984). Lymphocyte suspension density and viability were determined using a hemocytometer and trypan blue exclusion test. All lymphocyte densities and PFC counts were done in duplicate and the average of the two measures recorded.

Statistical evaluation

Data was analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) computer program. A Student t test was used to compare the mean values obtained from the two groups of flounder. Differences were considered significant at $P < 0.05$ level.

RESULTS

The mean condition factor [CF = Weight (g) / Length $(cm)^3$] of the NR flounder ($M = 0.0142$) was significantly higher ($P<0.05$) than the MC flounder ($M=0.01249$). There was no statistical difference in the mean hematocrit or serum cortisol between the two groups ($P >0.05$).There were significant differences in the mean PFC responses of flounder from the two sites (Table 1). The mean PFC response of the NR flounder was significantly higher ($P<0.05$) than the MC flounder when splenic lymphocytes were cultured for 9 day with TNP-LPS. The mean PFC response of the NR flounder was also significantly higher ($P<0.05$) than MC flounder when splenic lymphocytes were cultured for 14 day with TNP-KLH. There was no significant difference between the mean PFC responses when lymphocytes were cultured for 9 day with TNP-KLH.

Table 1
Modification of immune function of winter flounder caught in a minimally contaminated# and contaminated site@

Variable	Niantic River#	Morris Cove@
Condition Factor (g/cm^3)	0.0142 ± 0.000*	0.0124 ± 0.000
Hematocrit (%)	41.8 ± 1.4	39.1. ± 1.6
Serum cortisol (ng/ml)	77.87 ± 9.91	66.91 ± 14.45
	PFC / 10^6 lymphocytes	
9-day TNP-LPS	11.1 ± 2.2*	5.4± 1.2
9-day TNP-KLH	12.5± 4.5	4.5± 3.0
14-day TNP-KLH	17.7± 4.2*	6.8± 1.0

*Significantly higher than MC fish, $p<0.05$. Values are the mean of 15 fish ± S.E..

DISCUSSION

Many of the various methods available to evaluate the effect of xenobiotics on the piscine immune system have been reviewed by Weeks *et al.* (1992). Most immunological assays conducted on wild fish have utilized the non-specific immune response and particularly the alteration of macrophage functions (MacArthur *et al.*, 1984; Weeks *et al.*, 1990). The specific immune response is also affected by exposure to xenobiotics, but the majority of studies have been limited to cultured species (O'Neil, 1981; Bennett and Wolke, 1987; Anderson *et al.*, 1990) or *in vitro* toxicant exposure (Anderson *et al.*, 1989). The purpose of this study was to evaluate the primary *in vitro* plaque-forming cell assay as a potential technique for determining the presence of environmentally induced stress in fish. Although winter flounder have been selected as an important species for environmental monitoring (Murchelano and Wolke, 1991), few studies have been conducted to evaluate their immune systems. Lymphocytes obtained from winter flounder exposed

to contaminated sediments had a reduced ability to respond to both a T-cell independent antigen (TNP-LPS) and T-cell dependent antigen (TNP-KLH). A longer culture period (14 day) was required to obtain significant differences when TNP-KLH was used. In a previous study (Carlson *et al.*, 1993) prolonged *in vivo* administration of cortisol to winter flounder was shown to suppress the *in vitro* primary immune response as measured by the PHPA. The degree of this suppression depended on several factors, including the duration of elevated cortisol levels. The assay results were also affected by the type of antigen used (LPS or KLH) and the length of the culture period (9 or 14 day). Cortisol-mediated suppression of the PFC response was not statistically evident until day 10 post implantation using TNP-KLH and day 17 using TNP-LPS. This decline in lymphocyte responsiveness was paralleled by the increase in mean serum cortisol levels in the experimental fish which approximated the mean level observed in a group of diseased or disturbed flounder.The mechanisms involved in decreased responsiveness of the MC splenic lymphocytes were not investigated in this study. Although there was no statistical difference in mean cortisol levels of the two groups at the time sampled, a cortisol-mediated mechanism remains a possibe explanation. Our previous study (Carlson *et al.*, 1993) shows that the duration of cortisol elevation is a more important factor for decreased lymphocyte responsivness than the cortisol level. Genetic differences between the two groups of flounder cannot be ignored. The tendency of winter flounder to remain in a particular habitat makes it an ideal species for environmental monitoring but may also result in genetic differences between local sub-populations. The significantly higher condition factor of the NR fish may have resulted from naturally occurring differences in the two habitats. Water temperature and salinity were slightly higher at the NR site. The direct effect of temperature on the immune system of flounder has been investigated (Stolen *et al.*, 1984), although the effect of *in vivo* water temperature on the *in vitro* primary immune response has not been determined as well as water quality parameters such as dissolved oxygen, turbidity, and current velocity. These parameters as well as environmental contamination may have affected food availability and quality. As reviewed by Landolt (1989) and Blazer (1992), the immunosuppressive effect of nutritional deficiency has been observed in a number of species.In our studies, the level of contaminants in the fish was not determined and would be necessary to establish a direct relationship between contaminant presence and immunosuppression. Previous biological and chemical assessment of the two sites has been conducted (Gronlund *et al.*, 1991). The MC site was found to have a higher sediment content of aromatic hydrocarbons, polychlorinated biphenyls, and heavy metals (zinc, lead, copper, chromium, mercury, silver, and antimony). Winter flounder sampled at the MC site had a higher prevalence of histopathological changes and DNA alterations associated with contaminant exposure than flounder sampled at the NR site (Gronlund *et al.*, 1991). The primary *in vitro* PFC assay results suggest that the higher contaminant exposure noted by Gronlund *et al.* (1991) may also be the cause of the depressed specific immune response of MC flounder. The concept of using the primary *in vitro* PFC assay to evaluate contaminant-induced immunosuppression in flounder and to monitor aquatic environmental degradation is supported by this study.

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Chapter 13

Mechanisms of Immunosuppression by Aflatoxin B_1

Kaattari, S. L.[1], Adkison, M., Shapiro, D.[1] and M. R. Arkoosh[2]

Department of Microbiology
and
The Laboratory for Fish Disease Research
Oregon State University
Corvallis, OR 97331-3804

Current Addresses
[1]School of Marine Science, Virginia Institute of Marine Science
College of William and Mary
Gloucester Point, VA 23062

[2]Environmental Conservation Division, Northwest Fisheries Science Center
National Marine Fisheries Service, National Oceanic and Atmospheric Administration
2725 Montlake Blvd. E.
Seattle, WA 98112

ABSTRACT

Earlier observations in our laboratory revealed that embryonic exposure of rainbow trout to aflatoxin B_1 led to a long term alteration in the ability of rainbow trout to produce a normal antibody response. The two primary features of this alteration were: 1) a reduced capacity to produce a secondary antibody response to the T-dependent antigen trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), and 2) an altered fine specificity of this response. We have now focused on a detailed immunochemical and cellular analysis of this response in order to elucidate the possible mechanisms which may be involved.

Thus far, it has been found that : 1) aflatoxin-exposed trout experience a markedly delayed process of affinity maturation, 2) these fish also have a reduced capacity to produce serum immunoglobulins, and 3) leukocytes from these fish demonstrate a reduced capacity to respond *in vitro* to a T cell mitogen. Together these results suggest that aflatoxin appears to alter the capacity of the immune system to rapidly produce antibodies which have the highest affinity and specificity for antigen. Furthermore, these aflatoxin-induced alterations suggest that the effect may be mediated by a regulatory T cell dysfunction.

REVIEW OF STUDIES

Our research into the mechanisms of immunosuppression induced by embryonic exposure of rainbow trout (*Oncorhynchus mykiss*) to aflatoxin B_1 (AFB_1) has not only revealed some unique dysfunctions, but has led us through a number of revisions on how we view the functioning of the normal salmonid immune system.

Initially, our studies have employed trout which have been exposed by immersion to AFB_1 (0.5 ppm) for 30 min at the eyed-egg stage of development. These fish were permitted to mature to a size of 10 g before immunization with the T-dependent antigen, trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH). *In vitro* analysis of the antibody response capability began when these fish had attained a size of 100 - 300 g. Thus, as these effects (below) were manifested months after the short embryonic pulse of AFB_1, they must be considered long-term (Arkoosh and Kaattari, 1987).

We began our studies by examining the possible differences in antibody titer due to AFB_1 exposure during the primary and secondary antibody responses to TNP-KLH as described above. We observed that the primary antibody response was unaffected, while the secondary response was suppressed (Arkoosh and Kaattari, 1987; Arkoosh, 1989). We also found that lymphocytes procured from unprimed and primed (TNP-KLH) trout and challenged *in vitro* with either TNP-KLH or TNP-lipopolysaccharide (TNP-LPS) demonstrated a normal primary, but a decreased secondary antibody response. Normal controls revealed that, as with mice, priming with the T-dependent form of the antigen induced a memory state which can be elicited by either the T-dependent or independent form of the antigen (Arkoosh and Kaattari, 1991).

This effect of AFB_1 on the memory response prompted our exploration of other memory-associated phenomena. Of particular interest was the potential for an effect on affinity maturation. This is the process by which serum antibodies to a specific antigen increase in their average affinity over time after immunization. This process is enhanced during the secondary response to T-dependent antigen due to the contribution of somatic mutants to the high affinity pool of antigen-specific B lymphocytes (Berek and Milstein, 1988). Previous studies with other fish indicated that either affinity maturation does not occur or is quite limited in its expression (Makela and Litman, 1980; Litman *et al.*, 1980; Russell *et al.*, 1970; Clem and Small, 1967; O'Leary, 1980; Voss *et al.*, 1979). Initially, in our laboratory, the process of affinity maturation was examined by hapten inhibition of anti-TNP binding in an ELISA system (Arkoosh, 1989). In these studies, only one coating antigen concentration was employed which, retrospectively, did not permit sufficient resolution of the affinity of all antibody subpopulations (Gaya *et al.*,1986). Thus, these studies revealed that virtually no affinity maturation occurred during the antibody response in either normal or AFB_1-exposed trout.

In order to more fully analyze the antibody responses, fine specificity analyses of the antisera were conducted. In these analyses, relative association constants (Krel values) were determined (Pressman and Grossberg, 1968) for each antiserum. Krel values are relative measures of an antiserum's ability to bind a heterologous hapten as compared to the homologous form to which it was induced. Decreased Krel values indicate increased specificity for the inducing hapten. It was felt that any slight differences in binding site architecture that develop within the pool of antibodies could be more easily detected by such analysis than by the previously described ELISA. These fine specificity studies have demonstrated that: 1) the normal secondary response does change in its overall specificity, when compared to the primary response and 2) this secondary shift was distinctly

different in AFB_1-exposed trout when compared to their normal siblings (Arkoosh, 1989). We demonstrated that the secondary response of normal trout exhibited a decrease in the Krels for dinitrophenol, dinitrophenyl-γ-caproic acid, dinitrophenyl-γ-butyric acid as compared to the primary response. However, among the AFB_1-exposed fish, decreased Krels were not observed for dinitrophenol, but observed for dinitrophenyl-lysine and dinitrophenyl-phenylalanine.

These observations revealed two important features of the antibody response. First, it appeared that normal trout experienced shifts in fine specificity during the induction of a secondary response without a shift in overall affinity. This presented a dilemma in that a shift in specificity would logically be due to an increase in overall affinity of the antibody population. Regardless of the restrictedness of the antibody response, if there are B cells producing antibodies of different affinities then affinity maturation should eventually occur. Thus, if there is no affinity maturation, then not only must there be few B cells with different affinities, but they must all have virtually the same affinity. The fine specificity analyses indicated the opposite may be true. In all cases, when specific heterologs demonstrated a significant shift in their Krel values, it was a decrease. Thus, the antibodies appear to become more specific for the homologous hapten, an observation consistent with affinity maturation. Two possible explanations of these results were that affinity maturation was indeed occurring, but our original method of detecting it was too insensitive or, alternatively, there was no affinity maturation and it was simply coincidental that the Krel values were decreasing for heterologous haptens. As it will be demonstrated below, the former hypothesis proved correct.

The second feature revealed by the fine specificity analyses was that these shifts were distinctly different in AFB_1-exposed trout compared to control fish. This observation indicated that the expressed repertoire of antibodies was different in aflatoxin-exposed trout. Superficially perhaps, the most likely hypothesis which could account for such an occurrence would be that the dysfunction is related to an effect of AFB_1 or its metabolites on the B cell. One possibility could be that, as a genotoxic agent, aflatoxin was functionally inactivating genes encoding V regions of the antibody molecule. Past work in the murine system has indicated that in embryonic and neonatal mice, only D-proximal V genes are available for genetic recombinatorial events, and more distal V genes are not available until later in development (Perlmutter *et al.*, 1985). Thus, if similar patterns of V gene availability apply for AFB_1 DNA adduct formation, then upon embryonic activation of AFB_1 within the teleost anterior kidney (Al-Sabti, 1985), genetic lesions may be formed only within specific D-proximal V genes. Thus, only specific genes (and their resulting antibodies) would be affected. The possibility then arises that we may be able to identify the antibody specificities that are affected. This could be accomplished either by spectrotypic or idiotypic analysis. This form of analysis becomes feasible in certain teleosts including trout because the expressed repertoire of antibodies has been observed to be very restricted (Wetzel and Charlemagne, 1985; Richter and Ambrosius, 1988; Cossarini-Dunier *et al.*, 1986). Immunopurified trout antibodies to TNP have demonstrated less than 10 isoelectropherotypes, rather than the hundreds found in mammals. Absence of select idiotypic or isoelectric specificities should, therefore, be easily discernible. Upon identification of such idiotypes, the anti-idiotypic reagents can be used to screen expression libraries for the affected V, D, or J genes. Molecular probes for such genes could be produced and used to screen lymphocyte libraries of aflatoxin-exposed trout in the hopes of revealing any genetic lesions.

Alternatively, the alteration in the humoral memory response may be due to altered regulation of B lymphocytes. Since the T lymphocyte is a critical cell in the development of the memory response (Raff, 1970, Umetsu *et al.*, 1979; DeKruyff and Siskind, 1979), and since previous aflatoxin studies in a variety of animal species have revealed predominantly T cell dysfunctions (Pier *et al.*, 1977;

Reddy and Sharma, 1989), it is logical to pose that a T cell dysfunction may be indirectly responsible for the alterations in B cell function. This hypothesis would explain why aflatoxin has such pronounced affects on the magnitude and form of the secondary response.

We have begun to approach the role of T cells by first examining the effect of AFB_1 on T-dependent versus T-independent antibody responses. In order to accomplish this goal we have adapted a recently developed immunochemical technique for the assessment of antibody affinity. In this technique, graded coating antigen concentrations were employed in a hapten inhibition analysis (Gaya *et al.*, 1986), instead of a single coating concentration as was used in the affinity analysis described above (Arkoosh and Kaattari, 1991). The exposure of a constant concentration of antibodies to graded concentrations of antigen permits the capture and analysis of different antibody subpopulations based on their affinity. Each separate subpopulation is then assessed for affinity by hapten inhibition. Using this form of analysis, a more complete analysis of trout serum antibodies can be conducted, which reveals the process of affinity maturation.

Other studies in our laboratory have recently demonstrated that the affinity of antibodies for the T-independent form of antigen in normal trout do not attain the magnitude of those seen for the T-dependent antigen (5-fold less affinity). Since T-dependent responses in mice demonstrate tremendous affinity increases due to the accumulation of somatic mutations (Stenzel-Poore *et al.*, 1988), the altered fine specificity patterns seen during the secondary response may be due to such mutational processes. Additionally, in examining the affinities of antibodies generated in the aflatoxin-exposed trout, we have found that they do not express as high an affinity as is found in normal trout. This affinity difference is most significant early in the primary response; however, these values begin to approach the higher normal values late in the secondary response. A probable explanation of this phenomenon would be that it is due to dysfunction in the helper T cell population. If there is a deficit in functional T cells, T help would not be available to the degree that it is in normal fish. This deficit could conceivably affect the magnitude of the response, as well as the affinity of the response (less somatic mutants). Also, differences in the fine specificity of antibodies produced during the primary and secondary responses, which were observed in the initial studies, may be the result of this deficit. A reduced amount of somatic mutation could result in an impairment of affinity maturation; however, the gradual increase in affinity would indicate that some T help must be present, but not to the degree found in normal fish.

Most recently we have also observed that the total serum immunoglobulin levels in AFB_1-exposed trout are lower than in normal fish. This appears to be a dose-related phenomenon. The most dramatic reduction in serum Ig levels occurs with an embryonic exposure to 1ppm, with less differences after 30 min exposures to 0.5 and 0.01 ppm. What is particularly interesting about this study is that the aflatoxin appears to limit the capability of individuals to produce high concentrations of immunoglobulin. This lack of high serum Ig concentrations may be due to an inability to respond efficaciously to secondary exposures of environmental T-dependent antigens.

We have most recently begun *in vitro* cellular analyses of the lymphocyte function in normal and AFB_1-exposed trout. This is being accomplished by analyzing the mitogenic response of lymphocytes exposed to aflatoxin *in vitro*. This *in vitro* addition of aflatoxin suppresses mitogenic stimulation of normal lymphocytes by lipopolysaccharide, pokeweed mitogen, or phytohemagglutinin. There appears to be a slightly greater sensitivity among cells stimulated by phytohemagglutinin. However, it must be noted that these studies were performed during winter months, when immunoreactivity appears to be affected by endogenously high levels of plasma cortisol.

Of particular interest is the observation of a hypersensitivity to mitogens of lymphocytes from trout exposed to the lowest dose of aflatoxin. At this level, AFB_1 exposure has not been associated with the induction of tumors in trout. It is, therefore, possible that assessment of immunoreactivity may be a more sensitive monitor for toxicological or carcinogenic exposure than that of carcinogenesis. It is possible that reduction of a non-LPS or PWM responsive population may be responsible for the apparent hyperreactivity. Thus, it may be that within the population of cultured cells a greater proportion are B lymphocytes due to a reduction in the number of T lymphocytes. Alternatively, the hyperreactivity may be a manifestation of a regulatory cell dysfunction. For example, if T cells are generally more sensitive to the action of aflatoxin, perhaps T cells with greatest sensitivity (*i.e.* T suppressors: Ts) may be specifically eliminated at low AFB_1 concentrations. Specific removal of a Ts population by low dose toxicant exposure may then lead to hyperreactivity, not immunosuppression.

SUMMARY

Embryonic exposure to AFB_1 induces a long-term alteration in B cell function. This B cell dysfunction includes a lowered secondary response and a lower affinity antibody response. This effect may be due to a direct effect on the B cell, or an indirect effect of a dysfunctional T cell population.

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Chapter 14

Development and Use of Medaka as a Model for Immunotoxicity

Lorraine E. Twerdok[1], E. Maxine Boncavage-Hennessey[1], Robert A. Finch[2] and Henry S. Gardner[2]

[1]GEO-CENTERS, INC.
Frederick, MD USA

[2]U.S. Army Biomedical Research and Development Laboratory
Fort Detrick
Frederick, MD, USA.

ABSTRACT

Several species of teleosts, including medaka (*Oryzias latipes*), have been studied with respect to neoplastic responses to known carcinogens. Little information, however, is available concerning the responses of the immune system to environmental chemicals in the medaka. We have initiated studies to characterize immune organs and function in medaka, with the goal of using the medaka as a predictive model of immunotoxicity in vertebrates. Development of a teleost model for immunotoxicity is part of an integrated biological approach for assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. Medaka immune organs essential for leukopoiesis/erythropoiesis as well as cell morphology were examined. Cells isolated from the anterior kidney, spleen and whole blood were characterized with respect to nonspecific esterase activity, myeloperoxidase activity and acid phosphatase activity. *in vitro* phagocytic cell function following *in vitro* activation was assessed in primary cultures of pronephros adherent cells. Data collected to date characterizing the endogenous bacterial flora of medaka cultured in our laboratory is also presented.

INTRODUCTION

It has become increasingly evident over the last decade that the immune system is an important target organ for toxicity (U.S.Congress, 1991; Vos *et al.*, 1989; Dean *et al.*, 1982). Although immunotoxicology as a discrete field of study is advancing rapidly, the systematic study of immunotoxicity is complicated by the integrated nature of the immune system and the paucity of human exposure data available. Furthermore, there is currently no fully validated "battery" of short-term screening assays available to detect and assess potential immunologic toxicants (U.S.Congress, 1991; Luster *et al.*, 1992). A variety of test systems to assess immunocompetence is necessary due to the diverse and often overlapping mechanisms of defense utilized by the immune systems in response to immunologic challenge. Immunotoxicity can be manifested when one or more system(s) is compromised. Conversely, suppression or hyperactivation of one immune

function may have no adverse effect on overall host immunocompetence. Therefore, demonstration of *in vivo* adverse effects is critical in applied immunotoxicology research.

The overall goal of the research performed in our laboratory is to develop an integrated biological approach to assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. Existing components of this program for hazard assessment in the field include acute toxicity testing by ventilatory monitoring (Feder *et al.* 1992), teratogenicity assessment using frog embryos (Bantle *et al.*, 1991; American Society for Testing and Materials, 1991), carcinogenicity/tumor promotion assessment using medaka (Hawkins *et al.*, 1985, 1985; Battalora *et al.*, 1990), as well as Daphnia toxicity assays (Janssen and Persoone, 1993), and genetic toxicity assays using *in vitro* assays such as the Ames test (Ames *et al.*, 1975; Maron and Ames, 1983) and the CHO sister chromatid exchange assay (Wolff and Perry, 1974; Latt *et al.*, 1981). Due to recent advances in immunological research which demonstrate the importance of the immune system in many disease processes and chemically-induced toxicities, it is necessary to include detection of immunotoxicity into any integrated biological approach to hazard assessment. Thus, the specific aim of this research project is to develop and validate immunotoxicologic screening assays employing teleosts as the model system. The work presented here discusses our initial studies to characterize specific parameters of the immune system of the medaka (*Oryzias latipes*).

MATERIALS AND METHODS

Test species

Japanese medaka (*Oryzias latipes*) were reared in the laboratory in a flow-through well-water system maintained at 25°C with 16/8 hr light/dark cycle, and fed flake food (Tetramin®), brine shrimp and microworms. Five to seven month old medaka (500-600mg) which had received no treatment or stressful handling were used in all studies unless otherwise noted. Only fish which appeared healthy and active were used in these tests and groups were selected by age, without regard to sex.

Chemicals and Materials

L15 media, fetal bovine serum (FBS), glutamine and antibiotics were obtained from GIBCO (Grand Island, NY). Ninety-six well microtiter plates were obtained from Nunc, INC. (Naperville, IL). Luminol (3-aminophthalhydrazide) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained from Sigma Chemical Co., (St. Louis, MO).

Anterior kidney and spleen cell isolation

Fish were anesthetized with 200 mg/L MS-222 (tricaine methanesulfonate) and sacrificed by cervical transection. Anterior kidney and/or spleen were collected and pooled in 2-3 ml L15 media or fish physiological saline (FPS), then homogenized in glass/glass homogenizers.[Fish physiological saline consists of 110 mM NaCl (6.44 mg/L), 0.15 mM KCl (11 mg/L), 0.2 mM $CaCl_2$ (22 mg/L), $MgSO_4$ (12 mg/L), KH_2PO_4 (7 mg/L) and $NaHCO_3$ (10 mg/L) in distilled H_2O.] Cell suspensions were passed through glass wool to remove cellular debris and red blood cells and centrifuged at 350 xg for 15 min, washed twice with FPS, resuspended in 0.5 ml of FPS and counted using a hemocytometer. Cells from these whole organ homogenates were plated into 96-well microtiter plates at 2×10^5 cells per well and either used immediately (for special staining) or after 48 hr culture at 30°C in L15 media supplemented with 5% FBS, 2 mM glutamine, 100 IU/ml

penicillin and 100 µg/mL streptomycin. Cultured cells were washed after 24 hr to remove unattached cells and debris.

In later experiments, cells isolated from the anterior kidney were further purified by density gradient separation (Secombes, 1990). Three mL of the whole organ cell homogenate was layered onto 10 mL of a 51% Percoll® solution brought to isotonicity using 10X Hank's Balanced Salt Solution (HBSS), centrifuged for 30 min at 400 xg, and the fraction containing monocytes (a whitish band below the darker band of cellular debris at approximately the 50-60% fractions) collected. The monocyte-containing fraction was diluted with 5-10 mL FPS, washed twice and counted in a hemocytometer. Density gradient-enriched anterior kidney cells were then plated into 96-well microtiter plates at 2 x 10^6 cells per well and used immediately or incubated for attachment for 90 min at 25°C in L15 supplemented with 2% stock medaka serum (prepared as described below). After attachment, cells were either used immediately or cultured for 1.5, 24, 48 or 72 hr at 25°C in L15 supplemented with 5% FBS, glutamine and antibiotics. Cultured cells were washed twice at 1.5 and 24 hr to remove unattached cells and debris.

Blood cell and serum isolation

Anesthetized (MS-222, 200 mg/L) fish were cut into thirds and bled into 5 mL FPS + EDTA. Cells were centrifuged, supernatant collected, cell pellet resuspended in a small amount of FPS and the cells counted by hemocytometer for use in staining experiments. The supernatant containing fish serum was then further diluted with FPS to make a "stock" serum solution of 20 mL FPS (containing serum from 20 medaka). Assuming a recovery of approximately 10 µl serum per fish, the concentration of the stock serum solution was ≈ 1% serum in FPS (v/v). The solution was then filtered through a 0.22 µ filter and frozen at -20°C until needed for assays. Prior to use, stock serum was heated in a waterbath at 56°C for 20 min (DeKoning and Kaattari, 1992) to inactivate the complement.

Cell staining

Esterase; acid phosphatase, and myeloperoxidase (MPO) enzymatic activities associated with cells from the anterior kidney, spleen and blood were assessed by special staining techniques. Slides were prepared using ≅ 10^5 cells from each specified organ preparation using a cytocentrifuge. Slides were then stained for esterase activity [using two substrates,α-naphthyl acetate and/or naphthol AS-D chloroacetate (Sigma cat. # CP-1, Procedure No. 91)], acid phosphatase activity (Sigma cat. # CP-1, Procedure No. 181) or MPO activity (Sigma cat. # CP-1, Procedure No. 390).

Superoxide anion production

Superoxide dismutase-inhibitable extracellular and intracellular superoxide anion production was detected by assessing reduction of cytochrome *c* or nitroblue tetrazolium (NBT), respectively (Secombes *et al.* 1988; Jensen *et al.* 1991; Zelikoff and Enane, 1991; Secombes, 1990). Whole organ anterior kidney or spleen cell preparations were plated at 2 x 10^5 cells per well and assayed for superoxide anion (O_2^-) production 48 hr later. Attached cells were stimulated with 1 µg/mL 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and the amount of superoxide dismutase-inhibitable O_2^- produced was assessed 15 min after stimulation by spectrophotometric measurement in a microtiter plate reader at 550nm (Abs_{550}) or 620nm (Abs_{620}) for cytochrome *c* or NBT, respectively. The changes in absorbance were then used to calculate the amount of cytochrome *c* or NBT reduced

according to the method of Pick (Pick and Mizel, 1981; Pick *et al.* 1981). Results are presented as nmol cytochrome *c* or NBT reduced per 2 x 10^5 cells. Results are adjusted for nonspecific reduction of ferricytochrome *c* by subtraction of non-superoxide dismutase-inhibitable production. Cell preparations enriched for monocytes/macrophages by Percoll® separation and attachment to plastic were assayed for superoxide anion production by cytochrome *c* reduction after 0, 1.5, 24, 48 and 72 hr in culture. Cells were washed twice with HBSS, incubated with cytochrome *c* (C_f=1.6 mg/mL) with and without superoxide dismutase (SOD; C_f=50 µg/mL), and then stimulated with TPA (C_f=1 µg/mL) or solvent controls. Abs_{550} was measured every 15 min for three hr and results calculated as described above.

Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production following TPA-stimulation was assessed using peroxidase-dependent oxidation of phenol red (Secombes, 1990). Assay conditions were the same as those described for O_2^- production for whole organ preparations. For these studies, H_2O_2 production was not assessed in monocyte/macrophage enriched preparations.

Measurement of chemiluminescence (CL) responses. CL was monitored in freshly isolated anterior kidney cells in an ambient temperature liquid scintillation counter (Packard 2500TR) in the Single Photon Mode. Samples contained 2 x 10^6 anterior kidney cells and luminol (C_f=25 µM) in a total volume of 2 mL. Reactions were initiated by the addition of TPA (C_f=1 µg/mL) to the samples and monitored for 2 hr. Results are expressed as counts per unit time minus background. Data are expressed as peak cpm (maximum responses) and area under the curve (AUC; counts x min). Area under the curve was approximated using the trapezoid method.

Bacterial isolation and identification. Characterization of bacterial flora of presumed enteric, skin (mucus) and gill origin was accomplished by isolating bacteria from the water of aquaria containing 6 month old fish. Fish which appeared healthy and active were transferred into clean aquaria and held, without feeding, under static conditions. Seventy-two hr after the addition of fish, water samples were taken for bacterial isolation and identification. Approximately 500 mL aquarium water was filtered through a 0.22 µ filter and the filter was inverted and "streaked" onto tryptic soy agar (TSA). The filter was then removed and discarded and the plates were incubated at 25°C. After 48 hr, colonies of different macroscopic appearance were restreaked onto fresh TSA and incubated at 25°C. This process was repeated until pure cultures were obtained. These pure cultures were then sent out to commercial laboratories for identification by morphological and biochemical methods. Characterization of endogenous bacterial flora in intact fish was determined in 15 month-old medaka which had been used as a breeding population between 5 and 9 months of age. These animals did not demonstrate any external symptoms of disease, but had undergone significant handling- and age-related stress. Ten fish were euthanized with MS-222 (400 mg/L), necropsied aseptically and tissue samples from various organs including spleen, anterior kidney, liver, heart, lining of the peritoneal cavity or any macroscopic lesion (*e.g.* eye) were streaked onto TSA. Pure cultures of bacteria were isolated and identified as described above.

RESULTS AND DISCUSSION

The data presented in this paper are from initial experiments designed to develop a teleost model for immunotoxicity. This information will then be incorporated into an integrated biological

approach for assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. The initial steps in developing a teleost model for use in immunotoxicity screening assays is selection of species and characterization of immune organs from that species. We have selected the medaka, *Oryzias latipes*, for use as a teleost model of immunotoxicity for four reasons; (1) ease of culture and hardiness of medaka in general (Kirchen and West, 1969), (2) the genetics of medaka are well-characterized (Ijiri, 1987; Naruse *et al.*, 1988), (3) the susceptibility of medaka to chemical carcinogens is well-characterized and appears mechanistically analogous to mammalian chemically-induced carcinogenic processes (Gardner *et al.*, 1990; Van Beneden *et al.*, 1990; Hyodo-Taguchi *et al.*, 1985, 1989; Hawkins *et al.*, 1985,1988), (4) the small size of the fish will permit the use of large numbers of animals for laboratory or field exposure. It is anticipated that a large sample size will be necessary to detect toxicity at the concentrations of chemical contaminants found in the environment.

Characterization of the immune organs of medaka included cell yields and viability, staining characteristics for non-specific esterase, acid phosphatase and MPO activities, phagocytic cell function, and preliminary characterization of endogenous bacterial flora. Cell yields and viability as measured by trypan blue exclusion are given in Table 1. Enrichment of anterior kidneys for macrophages/monocytes decreased yields by 50-60% as compared to the whole organ cell preparation yields. Cell viability as measured by trypan blue exclusion was routinely ≥ 95% for anterior kidney preparations and ≥ 90% for spleen cells. Cell yields were not calculated for blood, *i.e.* cells collected from fish bled into FPS.

Table 1.
Average cell yields from anterior kidney and spleen of Japanese medaka

	Cell Yield* (# cells x 10^6/animal)	Viability (% cells excluding trypan blue)
Anterior kidney		
Whole organ	1.99 ± 0.46	95.0 ± 1.24
Enriched preparation	0.88 ± 0.01	97.9 ± 0.45
Spleen		
Whole organ	1.85 ± 0.46	90.0 ± 2.59

*Values represent Mean ± SEM, n ≥ 3.

Whole organ cell population differentials for non-specific esterase, acid phosphatase and MPO activities are given in Figures 1 and 2. As expected, many cells of head kidney and spleen origin were positive for esterase and acid phosphatase activities, indicating the presence of monocytic cells and those of lymphocyte origin, respectively. The percent of cells stained positively for esterase activity assessed using the double-staining technique (two substrates as opposed to a single substrate) was 59.4 ± 4.72 (values given are Mean ± SEM) and 50.9 ± 12.8 in anterior kidney and spleen cells, respectively; 14 .4 ± 2.81% and 8.02 ± 1.70 % of cells stained positively for acid phosphatase activity, respectively. While present, the percent of esterase and acid phosphatase positive cells was much lower in cells found in the blood. Interestingly, a considerable number of cells were MPO positive, 24.7 ± 1.27 %, 20.8 ± 16.2 % and 3.50 ± 2.29 % from anterior kidney, spleen and blood, respectively, presumably indicating a significant number of neutrophils in the

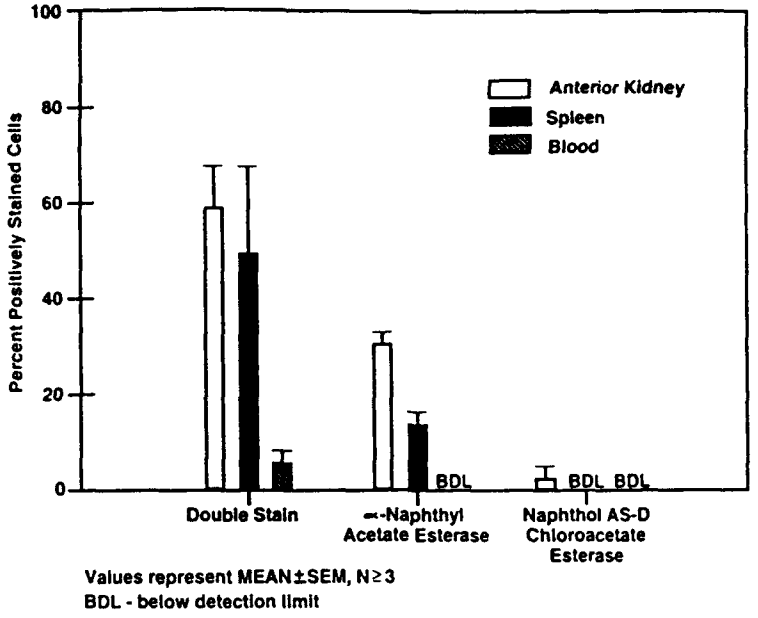

Figure 1. Esterase staining characteristics in medaka whole organ preparations.

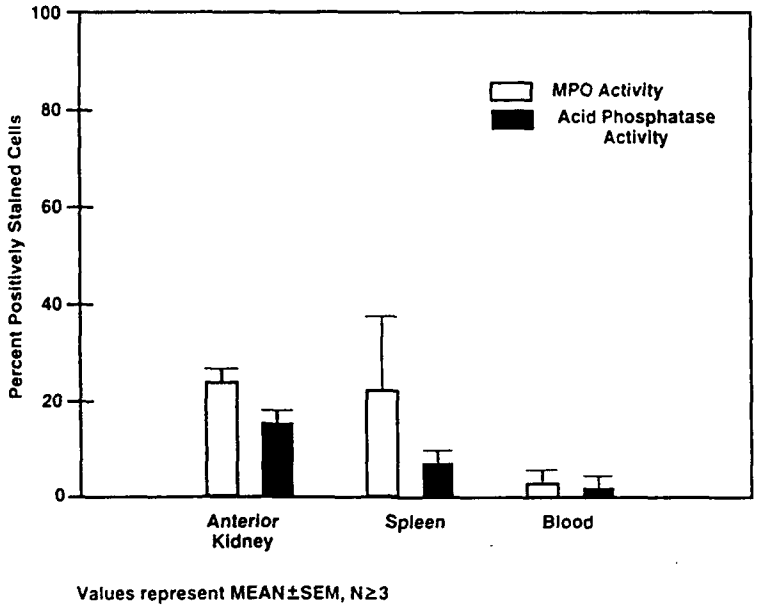

Figure 2. MPO and acid phosphatase staining characteristics in medaka whole organ preparations.

medaka. We plan to investigate these findings further with assessment of enzymatic activity in cytosol preparations. It should be noted that a large amount of variability in staining differentials was observed in the spleen as compared to the anterior kidney.

Measurement of reactive oxidant generation was assessed *in vitro* by measurement of reactive oxidants following stimulation by phorbol ester. Initial experiments demonstrated measurable

Table 2
Oxidant generation by TPA-stimulated anterior kidney and spleen cells of japanese medaka[a]

	ANTERIOR KIDNEY[b]	SPLEENb
Superoxide anion production[c] (nmol reduced/2 x 10^5 cells plated)		
Cytochrome *C*	0.536 ±0.137	0.03 ±0.001
Nitroblue tetrazolium	0.513 ±0.013	BDL[d]
H_2O_2 production (nmol reduced/2 x 10^5 cells plated)		
Phenol red	0.426 ± 0.030	0.090 0 ±008

production of superoxide anion and hydrogen peroxide by activated anterior kidney or spleen cells from whole organs (Table 2). Luminol-amplified oxidant-dependent generation of chemiluminescence was demonstrated in TPA-stimulated anterior kidney whole organ preparations (Table 3).

Table 3.
Quantitation of the luminol-amplified chemiluminescent response from TPA-stimulated anterior kidney cells

SOURCE OF CELLS	AUC[a] CPM [(cpm x min) x 10^8]	PEAK (cpm x 10^6)
Whole Organ Preparation	3.22 ±0.58[b]	6.03 ± 1.33

[a]AUC = area under the curve as estimated by the trapezoid method.
[b]values represent MEAN ± 5 , SEM, n ≥ 5.

In an effort to increase the size and reproducibility of the oxidant response in anterior kidney-derived cells, the whole organ preparations were enriched for monocyte/macrophage content by density gradient separation followed by attachment to plastic. Production of superoxide anion after stimulation was then assessed immediately after Percoll® separation and 1.5, 24, 48 and 72 hr after attachment to tissue culture vessels. It was hypothesized that activity would increase with culture time as monocytes differentiated into macrophages and the data presented in Figure 3 supported this hypothesis. There was no detectable production of superoxide anion within 180 min after addition of TPA at 0 hr post-attachment (immediately after Percoll® separation) or 1.5 hr post-attachment; production peaked at 24 hr post-attachment and returned to baseline by 72 hr post-attachment (Figure 3). Data from 72 hr post-attachment assays are not given. At 24 hr and 48 hr

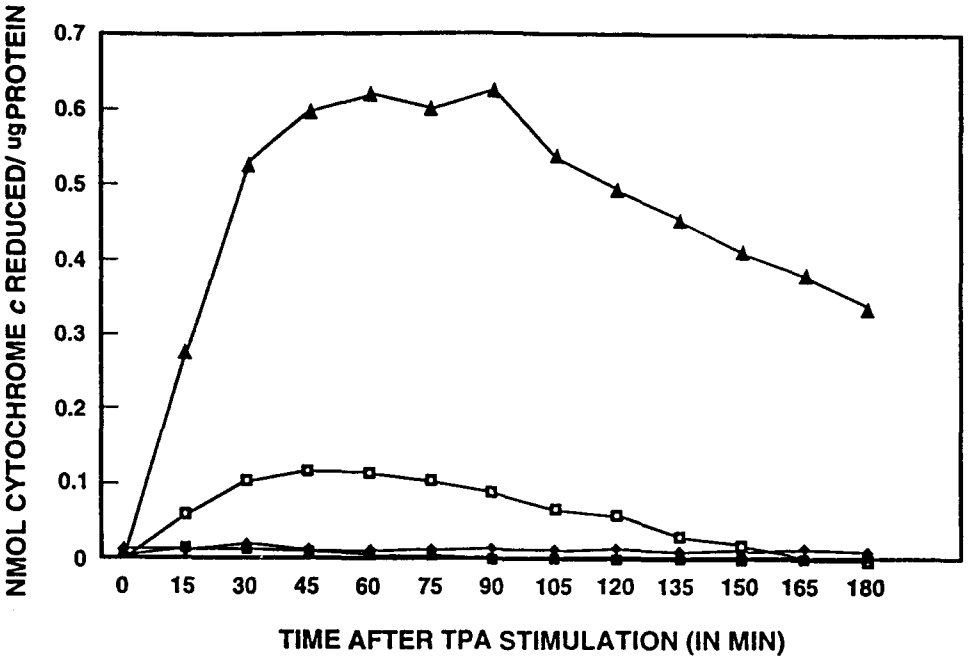

Figure 3.TPA-stimulated SOD-inhibitable superoxide anion generation in macrophage-enriched anterior kidney preparations was monitored for 180 min at various times after cellular attachment to plastic in culture: 0 hr post-attachment (no attachment) ■, 1.5 hr post-attachment ◆, 24 hr ▲, 48 hr post-attachment □, (72 hr post-attachment data not shown).

post-attachment, production of superoxide anion in monocyte/macrophage enriched cells peaked 45 min to 1 hr after stimulation with TPA. These data suggest that a significant number of "resting" or "resident" monocytes/macrophages reside in the anterior kidney of medaka which can be matured via attachment to plastic to a mature state responsive to TPA stimulation as demonstrated by a subsequent respiratory burst. Based on change in absorbance data, the values observed for superoxide anion production by medaka anterior kidney cells are comparable to those obtained for rainbow trout by other investigators (Secombes *et al.*, 1988; Secombes and Chung, 1988). However, recent data generated in our laboratory (data not shown) suggest that stimulated trout macrophages generate greater absolute quantities of superoxide anion than medaka-derived macrophages. Further characterization and optimization of oxidant generation in medaka cells is in progress.

The final parameter characterized was identification of endogenous bacterial flora. Since future experiments will incorporate an *in vivo* pathogenic challenge or *in vivo* immunogenic challenge for primary antibody response, it is necessary to characterize the composition of the normal bacterial flora in the test species. It should be noted, however, that the composition of bacterial flora can change significantly with water and food sources, and the results presented here are most likely location- and culture-specific. No bacteria were isolated on tryptic soy agar following repeated culturing of processed water before entry into culture tanks. *Acinetobacter, Pseudomonas, Enterobacter* and *Klebsiella* were isolated from the culture water of 6 month old fish (Table 4). Water borne bacteria were assumed to be primarily of enteric, scale/mucus and gill origin, since no organisms were isolated from culture water before the introduction of fish. Bacteria isolated from the internal organs of 15 month old medaka included *Aeromonas, Pleisonmonas* and *Pasteurella* species (Table 5). These data represent preliminary studies, and a more comprehensive characterization of the endogenous flora of medaka from our facility, as well as from other culture facilities, is planned.

Table 4.
Bacterial flora of medaka exterior surface and enteric origin[a]

Acinetobacter calcoaceticus (var. *anitratus*)
Acinetobacter calcoaceticus (var. *lwoffi*)
Pseudomonas species (morphology one)
Pseudomonas species (morphology two)
Aeromonas caviae Enterobacter cloacae
Klebsiella pneumoniae
Pseudomonas species

[a]Bacteria were isolated on tryptic soy agar from water from aquaria containing 6 month old fish held, without food, for 72 hr under static conditions.

Table 5
Results of bacterial cultures from internal organs of 15 month old medaka

Sample Site	Organism(s) Isolated	Antibiotic Sensitivity	
		Resistant	Sensitive
Posterior abdominal wall	*Aeromonas hydrophilia*	ampicillin	trimethoprim/ sulfamethoxazole
		tetracycline	cefamandole
		chloramphenicol	cifonitin
			ciprofloxacin
			gentamycin
			tobramycin
			ticarcillin
	Acinetobacter lwoffi	none	all of above
	Pleisomonas species	none	all of above(chloramphenicol not run)
Eye	*Pasteurella species*	tetracycline chloramphenicol	all of above
Anterior kidney	*Pasteurella species*	tetracycline	all of above
Spleen	*Pasteurella species*	tetracycline	all of above

Note: anterior kidney and spleen samples were from the same fish

The purpose of this work was to begin to characterize the medaka immune system with the goal of using this particular fish species for immunotoxicity hazard assessment of contaminants in the environment. *In vitro* generation of reactive oxidants by anterior kidney monocytes/macrophages following phorbol ester stimulation was analogous to similar experiments in rainbow trout, suggesting that the medaka may provide a relevant model for assessment of immunotoxic hazard.

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Chapter 15

Macrophage Aggregates as Indicators of Environmental Stress

V.S. Blazer[1], D.F. Facey[2], J.W. Fournie[3], L.A. Courtney[3] and J.K. Summers[3]

[1]National Biological Survey
1700 Leetown Road
Kearneysville, WV 25430

[2]Department of Biology
Saint Michael's College
Winooski Park
Colchester, VT 05439

[3]Center for Marine and Estuarine Disease Research
U.S. Environmental Protection Agency
1 Sabine Island Drive
Gulf Breeze, FL 32561

ABSTRACT

Although numerous investigators have noted changes in macrophage aggregates (MA) when comparing contaminated and reference sites, there is little information available as to the factors which may affect the accumulation of these structures. We are interested in assessing their usefulness as indicators of environmental stress as well as in examining the effects of other factors such as fish species, age, and parasite load. The use of splenic and hepatic MA parameters was evaluated in both estuarine and freshwater systems. Estuarine fish were collected during the Environmental Protection Agency's Environmental Monitoring and Assessment Program (EMAP). A variety of fish species are collected from numerous sites every year for histopathological analysis. At each site physical, chemical and biological data, including 110 sediment and 55 tissue contaminant levels, bottom dissolved oxygen, benthic and fish abundance, are also collected. Using image analysis we measured the number and mean size of MAs per mm^2 of splenic tissue. From these analyses the amount of tissue occupied by MAs was calculated. An increase in MA number and percent of tissue occupied by MAs was observed in most species from contaminated sites. MAs of gafftopsail catfish were the best indicators of elevated tissue contaminants while both catfish and spot MAs were good indicators of elevated sediment contaminants.

Yellow perch, brown bullheads and rock bass were collected from five sites in Lake Champlain, VT-NY. Three of these sites are known to have sediment contaminant problems. In this study fish were aged. When comparing fish of similar ages, MAs of rock bass and brown bullheads were good indicators of sediment contamination. Significant differences in yellow perch MA parameters were

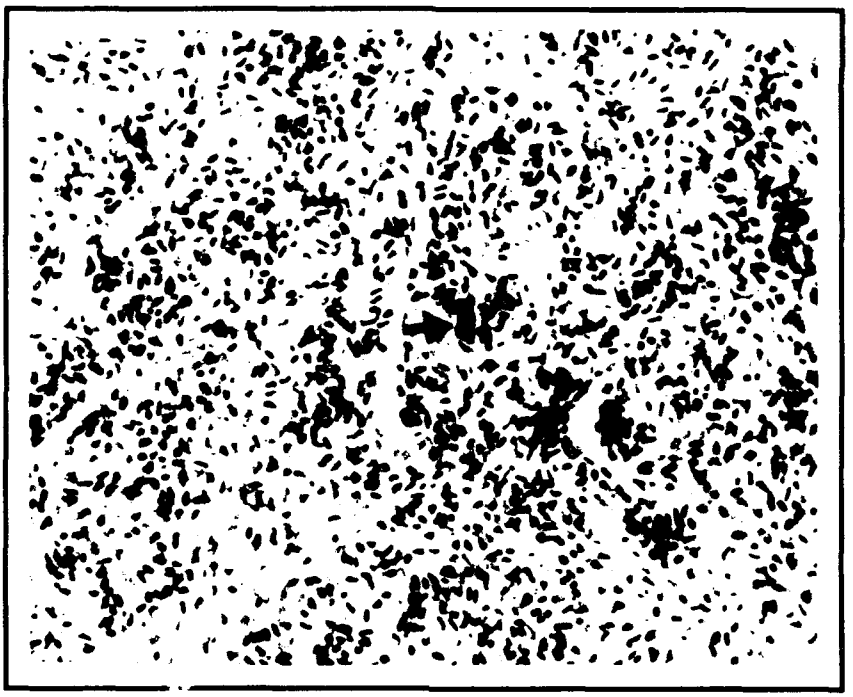

Figure 1. Macrophage aggregates in a rainbow trout spleen (arrows). These structures are small, irregular and black.

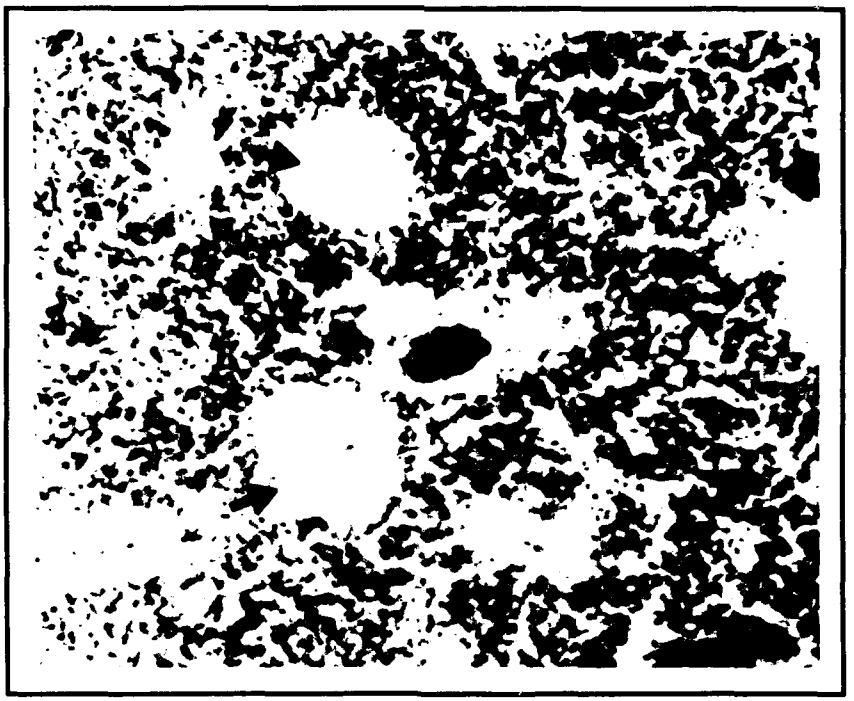

Figure 2. Macrophage aggregates in the spleen of yellow perch (arrows). These structures are larger, more regular and contain more yellowish-brown pigment.
Magnification on Figures 1 and 2 = X250

not noted between reference and contaminated sites. Correlations between age and MA number (r = 0.69) and percent of tissue occupied by MAs (r = 0.70) were observed in yellow perch collected at the reference sites. However, this relationship was not evident at the contaminated sites, indicating some factor was influencing their accumulation. In this study we also evaluated mean intensity (as a measure of pigment content) and shape factor of MAs. Preliminary results indicate measurement of these parameters may be useful in increasing the specificity of these structures.

INTRODUCTION

In recent years there has been an increasing awareness of environmental degradation and it's adverse effects on fish populations. This awareness has led to many federal and state monitoring programs which often include fish health assessment. These developments have also stimulated the search for biomarkers. Biomarkers are defined as morphological, physiological, pathological or molecular changes which reflect exposure to environmental contaminants (Huggett *et al.*, 1992). One potential biomarker is macrophage aggregate number/size. Macrophage aggregates (MAs) in fish have recently been reviewed by Wolke (1992).

MAs are focal accumulations of macrophages found in the spleen, head kidney and sometimes liver of fishes. MAs contain three pigments which can be visualized with a Perl's iron stain: ceroid/lipofuscin appears yellowish-brown, melanin appears black and hemosiderin stains blue. In most fish, individual aggregates contain two or three of these pigments. The aggregates range from small, black, irregular structures found in the salmonids (Figure 1) to the larger, yellowish-brown, more organized structures seen in many higher teleosts (Figure 2).

A number of functions have been attributed to these structures. It is believed they function in the centralization of foreign material and cellular debris. Early reports suggested the centers were repositories for material which was metabolically inert or which needed to be recycled (Roberts, 1975). Other literature suggests they are more dynamic structures. They appear to have a role as primitive lymph nodes or germinal centers and hence are important in the immune response (Ellis *et al.*, 1976; Ferguson, 1976). Immunization led to an increase in the size and number of MAs in goldfish, *Carassius auratus* (Herraez and Zapata, 1986). In addition, they have been shown to be important in iron metabolism and recycling (Agius, 1979).

There are many factors which may affect the accumulation of these structures, including age (Brown and George, 1985; Blazer *et al.*, 1987), fish size, nutritional status and infectious disease (Agius 1979, 1980; Agius and Roberts, 1981; Wolke *et al.*, 1985a,b). Changes in MA parameters in relation to environmental contamination have also been noted by a number of investigators (Poels *et al.* 1980; Bucke *et al.*, 1984; Kranz and Peters, 1984; Wolke *et al.*, 1985b; Benyi *et al.*, 1989; Spazier *et al.*, 1992). Both field and laboratory studies are needed to understand fully the relationship of MA number, size and pigment content to various environmental contaminants. There are many unanswered basic questions regarding MA formation (stimuli, kinetics, relationship to immune response), differential accumulation of MAs in different tissues, and correlation of changes in MA parameters with parasite load, pathogen or other antigenic exposure, which need to be answered.

We are interested in addressing the following questions to better evaluate these structures as biomarkers.

1). Is one type of fish species, due to food habits, habitat preference, home range, or other factors, better than another for use in assessing contaminant effects in a particular ecosystem?

2). Is age an important factor to consider when using these structures as biomarkers?

3). Do changes in MAs correlate with sediment or tissue contaminant levels in particular ecosystems?

4). Are there factors, other than size and number, which could provide more specificity?

5). Are splenic, hepatic or kidney MAs better indicators? Or will certain contaminants affect MAs in a particular organ and can this be used to enhance their specificity?

We have been involved in a number of studies in the past year in which we were able to collect tissues for macrophage aggregate analysis. Two of these studies will be presented in this paper. A project to assess fish health at a number of sites in Lake Champlain, Vermont/New York allowed us to collect tissues from a number of freshwater fishes. The second study, a collaborative effort with the Environmental Protection Agency's Environmental Monitoring and Assessment Program (EMAP), allowed us to study a number of estuarine fishes in the Gulf of Mexico.

METHODS AND MATERIALS

Lake Champlain Study

Lake Champlain is a glacially-formed lake bordering Vermont and New York, USA and Quebec, Canada. The lake flows slowly north and drains into the St. Lawrence River. It is approximately 100 miles long and 12 miles wide at the widest point and is a complex ecosystem divided into five distinct subbasins interconnected by narrow and shallow passageways. Concern about water quality of Lake Champlain and its tributaries has prompted numerous studies addressing many aspects of the ecosystem. To our knowledge fish health has not been previously addressed.

Selection of sampling locations was based on the results of sediment analyses begun in 1991 by the Water Resources and Lake Studies Center, University of Vermont. This survey of 30 sites revealed that several sites were contaminated with polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and some trace metals. The two reference sites in our study, Malletts Bay (A) and Shelburne Bay (C), did not have detectable levels of the organic contaminants assessed in the current monitoring program. However, the site in Malletts Bay is close to the inflow of the Lamoille River and contains elevated levels of arsenic, nickel, and manganese. Shelburne Bay is a somewhat eutrophic area also subject to agricultural and suburban run-off. The three contaminated sites each represent different types of problems. The Burlington Harbor site (B) is primarily impacted by sewage discharge and storm water runoff from the city of Burlington, VT. Cumberland Bay is an area adjacent to the city of Plattesburgh, NY. The collection site (D) contains an old industrial sludge bed and is adjacent to a former oil barge loading dock. Years of industrial dumping has resulted in a black, oily sludge mixed with lake sediment. Site E, the mouth of Ticonderoga Creek, was an area impacted by the discharge of a paper mill which has since moved.

Yellow perch (*Perca flavescens*) were collected at all five sites, whereas brown bullhead (*Ameiurus nebulosus*) were collected at only two sites (A and D). Fishes were collected either by electroshocking (sites A,B,D,E) or trawling (site C), anesthetized, weighed, measured, sex determined, gross lesions recorded and either spines (brown bullheads) or scales (yellow perch) removed for aging.

Pieces of liver, spleen, hind and head kidney, gill, gastrointestinal tract and any tissue containing gross lesions were fixed in 10% neutral buffered formalin. Skinless fillets were collected, kept on ice in the field and subsequently frozen. Composite samples of five individuals of each species from each site were analyzed for trace metals and organic contaminants by the State of Vermont's Agency of Natural Resources Water Quality Division.

Fixed tissues were shipped to the National Fish Health Laboratory - Leetown, Kearneysville, WV and processed for histopathological examination. Slides were stained with hematoxylin and eosin (H and E), periodic acid Schiff (PAS) and Perl's iron method (Luna 1968).

Macrophage aggregate data were collected using the Jandel MOCHA^{TM} system. Macrophage aggregates were measured in square pixels and then converted to square microns. The mean number of MAs per mm^2 tissue, the mean size (μm^2) and the percent of tissue occupied by MAs were evaluated for spleens from all fishes. In addition, we analyzed hepatic MAs in the brown bullheads and included mean intensity and shape factor of hepatic MAs. The shape factor compares a given image to a perfect circle. The shape factor of a circle is 1; hence values between 0 and 1 are obtained. Average intensity is based on the gray values of a color image which has been converted to monochrome. Black has a value of 0 and white a value of 256. Hemosiderin and ceroid/lipofuscin have ranges of gray values which can be obtained for each image.

EMAP-Estuaries Study

Data for gafftopsail catfish (*Bagre marinus*), spot (*Leiostomus xanthurus*) and pinfish (*Lagodon rhomboides*) are presented here as a comparison with the freshwater situation. Details of the EMAP-Estuaries program in the Louisianian Province and contaminant results are presented by Summers *et al.* (1994 a,b). This program provides us with vast amounts of data with which to compare our macrophage aggregate results. EMAP-Estuaries is a division of the overall EMAP program specifically designed to determine and monitor the ecological conditions of estuarine resources throughout the United States. This is accomplished by measuring a number of indicators representing water quality, benthic and fish community characteristics, and sediment quality at selected sites. Water quality indicators include instantaneous and continuous dissolved oxygen, temperature, salinity, and pH, as well as stable isotopes, turbidity, nutrients, and chlorophyll. Benthic and fish community characteristics include abundance, biomass, length, species composition, gross lesions, histopathology, and tissue residues for 55 contaminants (fish only). Sediment quality indicators include 110 sediment contaminants, sediment toxicity bioassays (amphipod, mysid and penaed shrimp, and polychaete), total organic carbon, acid volatile sulfides, percent silt-clay content, and grain-size distribution. Unfortunately, because of the nature of the program, there are no true "reference" sites. We hope, however, that by using the data collected for a number of years we can compare sites with various contaminants and begin to see trends as to which contaminants consistently correlate with increased (or decreased) numbers of MAs.

Histological sections of spleens, cut at 6μm, were stained with Harris's hematoxylin and eosin or Perl's prussian blue method (Luna 1968). Occurrence of MAs was assessed by measuring MA number and size (area) in three random 10X fields per spleen using computer-based image analysis (MicroComp^{TM} Integrated Image Analysis System, Particle Analysis). For each fish the mean number of aggregates per mm^2, mean size (μm^2) of the aggregates, and percent of tissue occupied by MAs were calculated. Statistical comparisons using SAS (SAS 1985) were made using data

from all fish from each site and, if significant differences in length were noted, subsets of data from fish of similar lengths from each site.

RESULTS

Lake Champlain Study

The sediment contaminant data for the five sites studied in Lake Champlain are presented in Table 1. All sites had measurable levels of a number of trace metals. Sites B and D had higher concentrations of cadmium, copper, lead and zinc than the other three sites. Site E had the highest level of chromium. The reference sites, A and C, did not have measurable levels of organic contaminants. Sites B, D and E had levels of PCBs and PAHs with site D having the highest concentration of both contaminant types.

Table 1
Sediment Contaminant Data from Lake Champlain[1].

Contaminants	Site A Malletts Bay	Site B Burlington Harbor	Site C Shelburne Bay	Site D Cumberland Bay	Site E Ticonderoga Creek
Sediment Metals (μg/g wet weight)					
Cadmium	0.24	1.05	0.38	0.63	0.40
Chromium	31.30	38.96	33.89	17.30	51.50
Copper	30.31	55.27	37.62	110.90	33.61
Lead	31.88	115.11	51.79	77.90	31.54
Nickel	59.03	30.49	42.31	9.40	28.92
Zinc	115.18	169.67	147.27	369.20	111.45
Sediment Organics (ng/g wet weight)					
Total PCBs	N.D.*	20.0	N.D.	50000.0	94.0
Total PAHs	N.D.	794.0	N.D.	75000.0	2277.0

[1]A. McIntosh and D. Lester, University of Vermont, unpublished data.
* Not Detectable

Brown bullheads were collected at sites A and D. We compared only 3 and 4 year old fish and hence there was no significant difference in mean age (Table 2). There were statistical differences between the two sites for all splenic MA parameters. More and larger MAs were found in bullheads collected at site D and consequently a greater percentage of splenic tissue was occupied by MAs at this site. There were much fewer MAs found in liver than in spleens of brown bullheads collected at these two sites. Although there were double the number of MAs in the liver of bullheads from site D when compared to livers from fish collected at site A, this was not statistically different. Nor was the MA size or percent of tissue occupied by MAs significantly different between these sites. There were significant differences between the sites for both the shape factor and the mean intensity of hepatic MAs. MAs from fish collected at site A were more regular (closer to a perfect circle) and had a lower mean intensity. This mean intensity value was within the ceroid/lipofuscin range.

Livers from bullheads collected at site D had MAs which were more irregular and had a mean intensity value which was within the hemosiderin range.

Table 2
Results For Brown Bullhead Collected at Lake Champlain[1].

Parameter	Site A	Site D
n	9	9
Age (yrs)	3.3 ± 0.5	3.4 ± 0.5
Length (cm)	27.2 ± 1.7	28.3 ± 1.6
Splenic Macrophage Aggregate Parameters		
MA #/mm^2	19 ± 4[a]	26 ± 5[b]
MA size (μm^2)	1972.9 ± 1117.6[a]	3207.5 ± 1446.0[b]
% tissue occupied	3.5 ± 1.2[a]	8.3 ± 4.1[b]
Hepatic Macrophage Aggregate Parameters		
MA #/mm^2	2 ± 1	4± 3
MA size (μm^2)	949.7 ± 432.4	1220.8 ± 890.5
% tissue occupied	0.2 ± 0.1	0.5 ± 0.5
Shape Factor	0.70 ± 0.08[a]	0.51 ± 0.16[b]
Intensity	113.2 ± 9.5[a]	141.1 ± 15.2[b]
Tissue Contaminant Levels		
Heavy Metals (mg/g wet weight)		
Mercury	0.10	0.13
Zinc	6.60	5.40
Copper	1.50	<1.50
Organics (ng/g wet weight)		
PCBs	N.D.*	2455.0

[1] Data are presented as Means ± S.D. Those values followed by the same letter are not significantly different at $p \leq 0.05$.
* N.D. = Not detectable.

Yellow perch were collected at all five sites. Statistically significant differences were noted for splenic MA parameters among the five sites sampled. MA number, size and percent of tissue occupied by aggregates were higher at sites A and E while fish from sites B, C and D were similar. However, mean age for the various sites also differed (Table 3 and 4). Fish from sites A and E were significantly older than fish from the other three sites. Since an increase in MA parameters related more to age than to sediment contaminant levels, we compared age groups from the various sites. Data from the two reference sites (A and C) were pooled and data from the three contaminated sites (B,D, and E) were pooled for the age comparison (Table 4). Although in most instances values for the contaminated sites were slightly higher than those of the reference sites, the differences were not statistically significant for any of the age groups. It should be noted that at the reference sites both the number of splenic MAs and the percent of tissue occupied increases with age.

Table 3
Results For Yellow Perch Collected at Lake Champlain[1].

Parameter	Site A	Site B	Site C	Site D	Site E
n	21	11	20	20	23
Age	3.1 ± 0.7^a	1.6 ± 0.8^b	1.8 ± 0.9^b	2.0 ± 0.9^b	2.8 ± 0.7^a
Length (cm)	18.9 ± 1.5^a	16.0 ± 1.7^b	17.2 ± 1.8^b	16.5 ± 4.6^b	18.9 ± 2.1^a
Splenic Macrophage Aggregate Parameters					
MA #/mm^2	19 ± 7^a	12 ± 6^b	9 ± 5^b	9 ± 6^b	19 ± 7^a
MA size (μm^2)	1322.4 ± 553.6^a	820.4 ± 289.7^b	811.8 ± 437.7^b	892.0 ± 401.8^b	1468.2 ± 894.2^a
% tissue occupied	2.5 ± 1.4^a	1.1 ± 0.8^b	0.8 ± 0.7^b	$0.8 + 0.6^b$	3.1 ± 2.8^a
TISSUE CONTAMINANT LEVELS					
Heavy Metals (μg/g wet weight)					
Mercury	0.25	0.13	0.10	0.19	0.15
Zinc	7.60	7.10	8.50	5.70	7.20
Copper	1.90	1.70	<1.50	<1.50	1.80
Organics (ng/g wet weight)					
PCBs.	N.D.*	N.D	N.D	2324.0	N.D.

[1] Data are presented as means ± S.D. Those values followed by the same letter are not significantly different at $p \leq 0.05$.
*N.D. = not detectable

Table 4
Yellow Perch Splenic Macrophage Aggregates Compared by Age and Site.

Age	Age 1		Age 2		Age 3	
Sites	A,C[1]	B,D,E[2]	A,C	B,D,E	A,C	B,D,E
MA #/mm^2	7 ± 3	8 ± 6	14 ± 6	16 ± 8	15 ± 7	15 ± 6
% tissue occupied	0.6 ± 0.4	0.8 ± 0.8	1.4 ± 0.8	2.3 ± 2.2	1.7 ± 1.0	1.8 ± 1.1
Sample size	10	14	8	15	15	18

[1] Reference sites A and C were combined for these analyses.
[2] Contaminated sites B,D, and E were combined for these analyses.

Linear regression analyses with age as the independent variable and MA parameters as dependent variables were compared for the two groups - reference and contaminated. At the reference sites we found good correlation between age and MA number (coefficient of correlation, $r = 0.69$) and age and percent of tissue replaced by MAs ($r = 0.70$). MA size and age were not as highly correlated ($r = 0.49$). In yellow perch collected at the contaminated sites, splenic MA number ($r = 0.33$), MA size ($r = 0.15$) and percent of tissue occupied by MAs ($r = 0.14$) were all poorly correlated with age.

EMAP-Estuaries Study

Sampling site WB1 was located in Watson's Bayou, FL, CR2 in the Choctawhatchee River, FL, AL3 in Wolf Bay, AL, and TX4 in Lavaca Bay, TX. Bottom water temperature was similar among the sites. Dissolved oxygen (DO) ranged from a low of 1.6 at CR2 to a high of 6.3 at TX4. Many organic and inorganic contaminants were present in the sediment at each of the sites (Table 5). TX4 had the lowest levels of most organic contaminants, with moderate levels of benzo(a)pyrene, benzo(a)anthracene and total PAHs present. CR2 had the highest levels of arsenic, chromium and total alkanes, while WB1 had the highest total PAHs, DDTs, chlordane, PCBs, benzo(a)pyrene and benzo(a)anthracene.

Table 5
Selected sediment contaminant levels at four sites in the Gulf of Mexico where fishes used for splenic macrophage analyses were collected.

Site	WB1	CR2	AL3	TX4
Dissolved Oxygen	3.6	1.6	5.1	6.3
CONTAMINANTS				
Organics (ng/g dry weight)				
Total Alkanes	3,120.50	12,919.90	3,430.70	709.10
Total PAHs	2,142.64	396.98	234.32	578.28
Total DDTs	14.66	3.70	2.24	0.00
Total Chlordane	1.88	0.12	0.33	0.00
Total PCBs	73.54	16.66	14.51	14.29
Benzo(a)pyrene	81.54	6.94	4.58	29.68
Benzo(a)anthracene	88.67	6.66	5.47	20.94
Heavy Metals (µg/g dry weight)				
Arsenic	5.10	22.30	7.10	7.90
Chromium	32.00	107.00	46.00	62.00

MA analysis was performed on pinfish, spot, and gafftopsail catfish. Spot collected at the Choctawhatchee River, FL (CR2) and Wolf Bay, AL (AL3), were similar in length (Table 6). The mean lengths of pinfish collected at Watson's Bayou, FL (WB1) and Wolf Bay, AL (AL3) were significantly different (Table 6). Hence, data collected from all fish, as well as those from only fish of similar lengths, were analyzed for site comparisons. Lengths of catfish collected at AL3 and TX4 were significantly different (Table 6). Again, data for all fish, as well as data from only fish of similar lengths, were analyzed for site comparisons.

Significant differences were noted in image analysis-derived MA parameters among fish from the various sites. Number of aggregates, mean size and percentage of splenic tissue occupied were all significantly higher in spot collected at CR2 than in those collected at AL3 (Table 6). Catfish MA parameter measurements were all significantly higher in fish collected at AL3 compared with those collected at TX4; however, lengths were also significantly different. When catfish of similar sizes were compared the significant differences persisted (Table 6). In catfish and spot, fish with higher

Table 6
Results of macrophage aggregate measurements in three fish species from four sites[1]

Species	Site	n	Fish Length mm	# of MA per mm^2	MA size μm^2	%Tissue Occupied
Spot[2]	CR2	18	95.4±5.2[a]	38.5±6.2[a]	2394.5±512.6[a]	9.3±2.2[a]
	AL3	20	96.5±2.2[a]	7.0±1.5[b]	301.0 ±61.6[b]	0.2±0.1[b]
Catfish[2]	AL3	21	109.9±2.4[a]	18.1±2.2[a]	1851.9±489.1[a]	3.6±1.2[a]
	TX4	19	97.7±2.7[b]	5.2±1.2[b]	286.7 ±56.1[b]	0.1±0.04[b]
Catfish[3]	AL3	10	105.4±1.9[a]	18.3±3.6[a]	1814.4±479.4[a]	3.7±1.5[a]
	TX4	9	102.0±3.9[a]	5.3±2.1[b]	314.4 ±89.8[b]	0.2±0.1[b]
Pinfish[2]	WB1	37	115.4±6.4[a]	42.5±6.9[a]	1442.6±242.3[a]	6.0±1.1[a]
	AL3	16	83.1±2.2[b]	26.8±4.7[b]	751.7±130.1[b]	2.0±0.5[b]
Pinfish[3]	WB1	10	88.6±2.4[a]	35.7±7.7[a]	756.6±205.9[a]	2.8±1.2[a]
	AL3	10	86.0±1.3[a]	29.0±6.1[a]	814.0±177.0[a]	2.3±0.6[a]

[1] Data are presented as Means ± 95% confidence level.
[2] All fish analyzed
[3] Subset of fish analyzed.
[a] Values within a cell followed by the same letter are not significantly different at $p \leq 0.05$.

numbers of MAs also had significantly larger aggregates and hence significant differences were seen in the percentage of tissue replaced. Pinfish MA parameters in fish collected at WB1 were all significantly higher ($p \leq 0.05$) than those in fish collected at AL3; however pinfish collected at WB1 were also significantly larger. When ten fish of similar lengths were compared there were no significant differences between the sites for any MA parameters.

Contaminants were measured in the tissues of the various fish species at each station (Table 7). In general, heavy metals were low in tissues and catfish tended to have higher levels of most contaminants when compared to the other two species collected at the same site.

Table 7
Comparison of selected tissue contaminants in fishes from different sites

Site	AL3	CR2	TX4	AL3	AL3	WB1
Species	Spot	Spot	Catfish	Catfish	Pinfish	Pinfish
Organic Contaminants (ng/g wet weight)						
o,p-DDD	3.5	2.1	0.0	10.4	2.2	0.8
p,p-DDD	0.0	0.0	0.0	0.8	0.0	0.0
o,p-DDT	0.4	0.9	0.0	0.3	0.0	0.0
p,p-DDT	0.6	0.6	0.0	3.0	1.2	0.0
Chlordane	0.0	0.0	0.0	0.7	0.0	0.0
Transnonachlor	0.0	0.0	0.0	1.3	0.0	0.0
Endosulfan	0.0	0.0	1.4	0.0	0.0	0.0
Hexachlorobenzene	0.0	0.0	1.4	0.0	0.0	0.0
Toxaphene	0.0	0.0	160.0	0.0	0.0	0.0
Total PCBs	0.7	1.3	1.0	12.9	1.7	0.2
Total Chlorinated Pesticides	5.3	4.2	161.4	19.0	5.7	1.7
Heavy Metals (µg/g wet weight)						
Aluminum	*	*	*	*	8.0	0.4
Arsenic	*	*	*	*	<0.1	<0.1
Mercury	*	*	*	*	0.020	0.004
Selenium	*	*	*	*	0.1	0.0

*Not done

DISCUSSION

In the introduction of this paper a number of questions were listed that were important to address before macrophage aggregates can be used as biomarkers. The results of the two studies will be discussed in the context of those questions.

Is one fish species better than another for use in biomonitoring?

Intuitively, the answer to this question would be yes. Since many contaminants tend to accumulate in sediments, fish which live and eat in these contaminanted sediments would be expected to be more "environmentally stressed" than pelagic-feeding fish. Fish which are more migratory and have a larger home range would be less impacted by a particular site than those which stay in the area. Indeed, many of the previous studies on MAs, have used bottom-dwelling fishes such as winter flounder (*Pseudopleuronectes americanus*), dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) (Bucke *et al.*, 1984; Wolke *et al*,. 1985b; Kranz and Gercken, 1987; Benyi *et al.*, 1989; Gardner *et al.*, 1989; Payne and Fancey, 1989). Conversely, carnivorous fish may be greatly impacted by contaminants which accumulate through the food chain.

Results from the two studies presented here also suggest the answer to the question is yes. Splenic MAs in yellow perch (of similar ages), from contaminated sites and reference sites were not significantly different. Conversely, splenic MAs of brown bullheads from a contaminated site were significantly different than those from bullheads collected at a reference site. The food habits and behavior of these two fishes are very different. Perch are pelagic foragers and major food items include forage fish, amphipods, chironomids and trichopterans (Jansen and Mackay, 1992). Brown bullheads are benthic-living, bottom-feeding, omnivorous fish with a smaller home range than perch.

The three estuarine species compared also differ in a number of aspects. All three species are reported to migrate offshore in fall and return to the estuary in late winter and spring (Hansen 1969; Muncy and Wingo 1983). Food habits and habitat preference of the three species do differ. Pinfish are more pelagic, omnivorous grazers that feed on or near rocks, pilings and seagrass beds. Major food items include shrimp, mysids, vegetation and amphipods. Spot are omnivorous, benthic-feeding fish that feed on polychaetes, clam siphons, harpactocoids and amphipods. Copepods have been reported to dominate the gut contents of postlarval spot, whereas juvenile spot are considered opportunistic benthic carnivores (Stickney *et al.*, 1975; Hodson *et al.*, 1981). The gafftopsail catfish is an omnivorous, bottom-feeding fish over sand and mud flats. Major food items include organic detritus, microinvertebrates, crabs, and shrimp (Muncy and Wingo, 1983).

Significant differences in splenic MA parameters in both spot and catfish were noted between sites (Table 6). When similar-size pinfish were compared there was no significant difference in MA parameters between the two sites compared. So, this study also suggests that choice of fish species is important. As in the freshwater study, the more pelagic-living and eating fish did not show a measurable response in splenic MA number, size or percent of tissue occupied.

Is age an important factor to consider when using these structures as biomarkers?

Previous work has shown that MAs increase with age in rainbow trout (*Oncorhynchus mykiss*) spleen and kidney tissue (Aguis 1981), yellow perch kidney (Brown and George, 1985) and largemouth bass (*Micropterus salmoides*) liver and spleen (Blazer *et al.*, 1987). Our data from the Lake Champlain study support the hypothesis that age is an important factor to consider. Splenic MAs and the percent of tissue occupied by MAs in yellow perch from the reference sites were highly correlated with age. It is interesting to note that this correlation was not apparent at the contaminated sites . Hence, there is some effect on MA formation, even in species for which the splenic MAs do not appear to be good biomarkers .

Fishes were not aged in the EMAP-Estuaries study. However, we made an attempt to control for this factor by comparing fish of similar lengths. Based on published data, the gafftopsail catfish used in this study were probably all age 0 fish (Muncy and Wingo, 1983). Published data on age and growth indicate that most of the pinfish in this study were age 1; a few may have been age 0 (Hansen, 1969). Studies of spot growth at different ages indicate that spot in the Gulf of Mexico generally reach 120 to 140 mm in the first year so the spot in this study were probably age 0 fish (Hales and van den Avyle, 1989).

Do changes in MAs correlate with sediment or tissue contaminant levels in a particular ecosystem?

In the freshwater study only brown bullhead spleens showed significant differences in MA parameters. This increase in splenic MAs correlated well with higher sediment organic contaminant levels and higher tissue PCB levels. A previous study with brown bullheads in the Hudson River showed an increase in hepatic MAs at a site known to have high concentrations of PCBs and heavy metals, versus a reference site (Bowser *et al.* 1990).

The estuarine study showed mixed results regarding the relationship of sediment or tissue contaminants and MAs. Spot MA measurements were higher at the site with the highest sediment contaminant load. Spot at this site also had the highest tissue levels of total PCBs. However, they had slightly lower tissue levels of total chlorinated pesticides. Catfish collected at AL3 had higher tissue levels of DDTs, chlordane, transnonachlor and total PCB's compared to catfish from TX4; this finding correlated well with the high MA values. Yet, catfish at TX4 did have higher tissue levels of toxaphene, endosulfan and hexachlorobenzene, which may indicate these particular contaminants do not seriously affect MA formation or may actually cause decreases in MAs. The sediment contaminants significantly higher at AL3 were total alkanes; total DDTs, total chlordane and PCB's were only slightly higher. However, sediments at TX4 had higher levels of PAHs, benzo(a)pyrene, benzo(a)anthracene, arsenic and chromium.

An interesting observation is that low DO seemed to correlate with higher MA parameters. For all fish species, fish collected at the site with the lowest DO had the highest number of MAs. Further work needs to be done to determine if low DO itself can lead to increases in MAs or if the low DO is simply an indicator of environmental degradation.

There are few reports on the response of MAs to controlled (laboratory) contaminant exposures or of other field observations with which to compare our results. Chronic exposure to different types of crude oil caused an increase in splenic and kidney MAs in Atlantic cod, *Gadus morhua* (Khan and Kiceniuk, 1984). A similar increase of hepatic MAs was noted in plaice, *Pleuronectes platessa*, captured in the area of an oil spill, when compared to those captured at a reference site (Haensly *et al.*, 1982). Pierce *et al.* (1980) reported an increase in hepatic MAs in starry flounder, *Platichthys stellatus*, from the Duwamish River, when compared to a reference site. PCBs were the main contaminants identified in these fish (Pierce *et al.*, 1980). Research on winter flounder (*Pseudopleuronectes americanus*) from eight coastal embayments indicated the splenic area occupied by MAs was correlated with chemical contamination of sediments. PCBs, PAHs and trace metals measured at these sites all correlated with MA parameters. The number of MAs and their size were found to increase with increasing levels of benzo(a)pyrene (Benyi *et al.*, 1989; Gardner *et al.*, 1989). Conversely, there is one report of exposure to sediments contaminated with a petroleum source of PAHs causing a decreased number of hepatic MAs in winter flounder (Payne and Fancey, 1989). Water-borne chromium exposures led to an increase in MA number but a decrease in the mean size of the aggregates (Kranz and Gerken, 1987).

Much more field and laboratory research is needed to answer the above questions. Our results, for brown bullheads, spot and gafftopsail catfish, as well as results reported by other investigators, suggest that increases in tissue and sediment PCB levels do correlate with increased MAs. For brown bullhead and spot increased MA measurements corresponded to high sediment levels of PAHs. Gafftopsail catfish showed the opposite trend. These differences may reflect differential metabo-

lism of these compounds or differential responses to complex mixtures of contaminants. Correlations with other sediment or tissue contaminants may exist but will require further investigation.

Are there characterisitics, other than number and size, which could be measured to increase the specificity of MAs?

We believe that other measurable characteristics may increase the specificity. Two characteristics are currently under study are shape factor and intensity (or relative pigment distribution). Since shape factor measures how circular an aggregate is, we hypothesized that during exposure to chemicals which lead to tissue breakdown or rapid formation of MAs, a greater proportion of irregularly-shaped MAs may be found. We have only begun to test this hypothesis, however, hepatic MAs from brown bullheads at the contaminated site did have significantly more irregular MAs.

A second hypothesis is that changes in relative pigment distribution may be indicative of certain types of contaminant exposure. There are a number of literature citations which suggest this hypothesis is correct. Hemosiderin within hepatocytes has been shown to increase in channel catfish (*Ictalurus punctatus*) following chronic exposure to water-borne arsenic (Sorensen and Smith, 1981). Hemosiderosis has also been reported in fish exposed to crude oil (Khan and Kiceniuk, 1984; Khan, 1991; Khan and Nag, 1993). In addition, an increase in the density of the melanin pigment has been reported in fish exposed to chromium (Kranz and Gerken 1987).

In our study there was an increased hemosiderin content of the hepatic MAs in brown bullhead from the contaminated site. This is similar to the findings of Bowser *et al.* (1990) for brown bullhead from a contaminated site in the Hudson River. A considerable amount of field and laboratory work will be required to determine if these factors can be used to increase the utility of MAs.

Are splenic, kidney or hepatic MAs better indicators?

All three tissues have been used by various investigators in both field and laboratory studies. Unfortunately we are aware of no studies which compare the MAs in the various tissues. It has been speculated that the MAs in different tissues have different functions (Kranz and Peters, 1984). We only present data for hepatic MAs of brown bullhead. In this case there were many less aggregates in the liver than in the spleen. It may be that certain contaminants affect MAs in a particular tissue and, hence, a comparison of these structures in all three tissues may increase their specificity. Again, more research is needed to adequately answer this question.

CONCLUSIONS

The results of these studies support the use of macrophage aggregate parameters as biomarkers of contaminant stress. A common criticism concerning the use of MAs as biomarkers is that they are nonspecific and too many variables are involved in alterations of their parameters. However, as Wolke (1992) pointed out, no single biomarker will necessarily provide both general and specific information regarding environmental contamination. Nonspecificity may be of great value if MAs are to be used as first-line indicators. Variations in MA parameters may serve to warn of potential problems and be followed by more specific studies to identify the actual stresses or to establish cause-effect relationships. In addition, further studies on pigment content, shape factor and differential responses of MAs in different tissues may increase their specificity. Advantages to the

use of MAs are their availability, ease of measurement and, in many cases, they can be evaluated even in poorly fixed tissue which would not be usable for other histopathological evaluations. Both laboratory and further field studies are necessary to understand the formation of these structures and how environmental contaminants affect them.

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Chapter 16

The Red Drum, *Sciaenops ocellatus*, as an Environmental Sentinel for Warm Water Estuaries

Karen G. Burnett, Leslie K. Schwarz and SaraJane V. Carlson

Marine Biomedical and Environmental Sciences
Medical University of South Carolina
Charleston, SC 29412

ABSTRACT

To assess its potential as a sentinel species for marine environmental monitoring and assessment surveys in the southeastern United States, our laboratory has initiated studies of immunological health in the indigenous red drum, *Sciaenops ocellatus*. *In vivo* immunization studies have shown that this teleost fish develops relatively high and persistent humoral responses to model antigens and that these responses are sensitive to acclimation temperature. *In vitro* mitogen-dependent proliferation of peripheral blood leukocytes (PBL) exhibits similar sensitivity to rapid temperature shifts. Thus seasonal and tidal temperature fluctuations introduce a major variable into the use of immunological tests as a biomarker for environmental health. To begin assessing the impact of this variable, we have tested whether feral red drum at immunologically permissive temperatures develop humoral responses to indigenous bacteria. For this study serum samples and bacterial cultures of mucosal slime were taken from individual animals within a single wild population. In ELISA each serum exhibited a distinct specificity profile on the panel of bacterial isolates. Future studies will explore the effects of changes in environmental temperature on these anti-bacterial responses and compare humoral response profiles among red drum populations within microhabitats of the same and adjacent warm water estuaries.

INTRODUCTION

To be an effective sentinel species for a marine or freshwater habitat, a teleost fish should have three characteristics. First, the species should be indigenous to the watershed under study (Lower and Kendall, 1990). A sentinel which is an exotic or transgenic organism may bring real or perceived risks to an environmental health assessment program. Even if the risk can be minimized by caging or other artificial confinement, the additional stress may reduce reliability of the indicator test. Second, in order to reflect health status of a watershed, the sentinel species should be limited to well-documented areas of movement. While relatively easy to obtain for many freshwater species, such documentation is available for relatively few marine teleosts (Lower and Kendall, 1990). Third, the sentinel organism should have an easily monitored biological function (biomarker) which is sensitive to environmental perturbations. *In vitro* studies have documented

that several components of the immune system in teleost fish are sensitive to chemical contaminants (Anderson, 1990). However, with the exception of natural population surveys which have focused on macrophage function (reviewed by Weeks, 1992), very few field studies have assessed the effects of environmental pollutants on humoral and cellular immune function in teleost fish.

One reason for limited assessments of immunological functions in feral populations has been the inability to control or predict antigen exposures in these individuals. Robohm *et al.* (1979) partially resolved this problem by assessing natural serum titers in flounder against a panel of bacterial antigens, including isolates from the fish populations under study. Using a different approach, Arkoosh *et al.* (1991) measured immunocompetence in feral chinook salmon by testing *in vitro* primary and secondary immune responses to model T and B-cell antigens.

These studies suggested that the flounder and chinook salmon were appropriate environmental sentinels for cold water estuaries of the United States. Similarly, our laboratory has proposed that the red drum *Sciaenops ocellatus* may have considerable value as a sentinel species for warm water estuaries. *S. ocellatus* is indigenous to brackish and near-shore waters of the southeastern United States, ranging from the Chesapeake Bay to the Gulf Coast. The species tolerates a broad range of

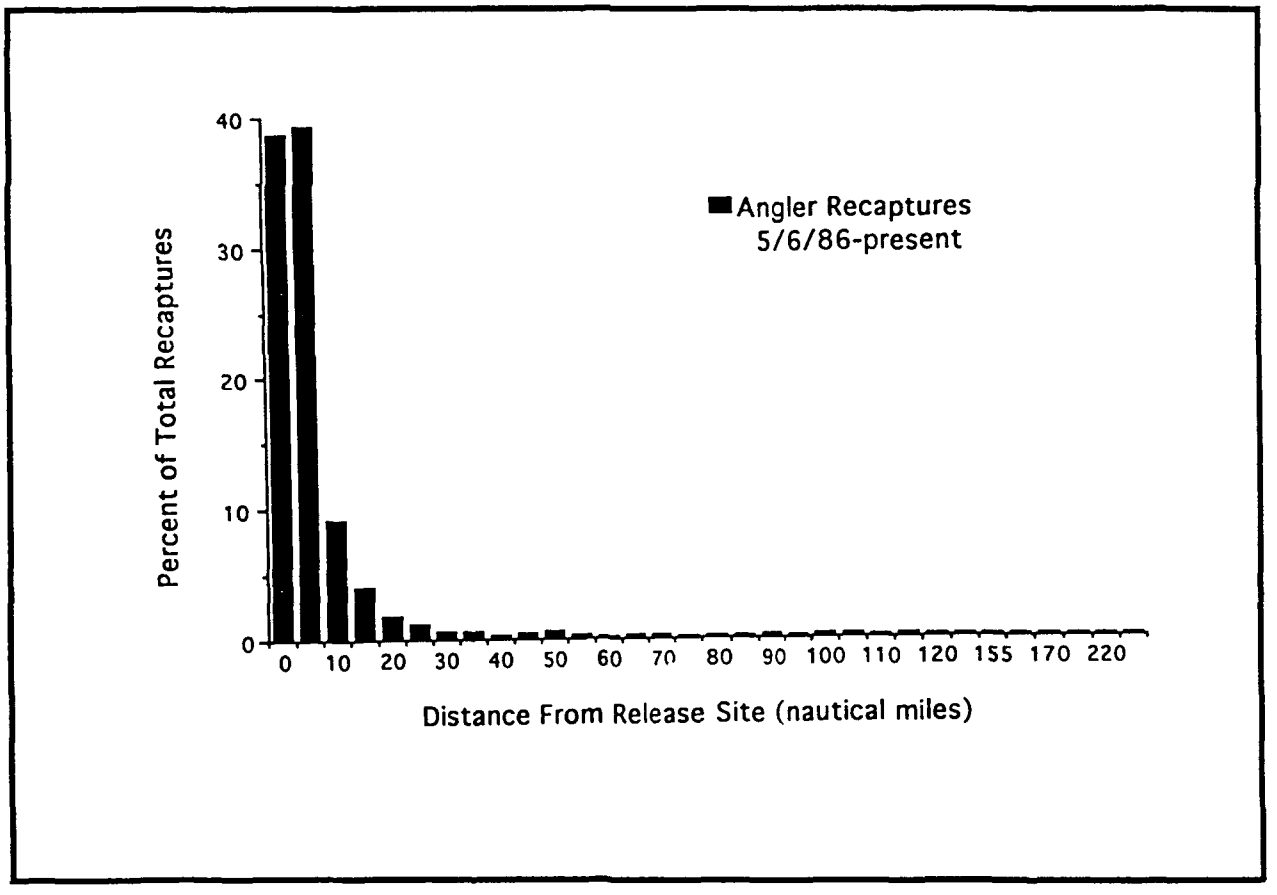

Figure 1. Recapture rates for tagged red drum. Sub-adult red drum were captured within several estuarine systems along the South Carolina coast, tagged and released on-site. This frequency histogram displays the distance of the recapture site from the release site for 1960 tagged animals obtained by anglers and reported to SCWMRD. Results are from 9486 animals tagged between May, 1986, and August, 1993. Where the same animal was recaptured multiple times, only the first capture distance is plotted. Data were obtained through the courtesy of the Inshore Fishery Group, South Carolina Wildlife and Marine Resources Department.

water temperatures from below 10°C to more than 31°C (Henderson-Arzapolo, 1992). Due to its popularity among recreational fishermen, the red drum has been a target for extensive tag and release studies by the South Carolina Wildlife and Marine Resources Department (SCWMRD). These workers have demonstrated that tagged feral, sub-adult red drum generally remain within a ten mile radius of release and do not migrate between estuary systems (Figure 1).

To exploit the potential of this species as a sentinel organism, we are developing assays to monitor immune function in the red drum. The current work documents the temperature sensitivities of certain *in vivo* and *in vitro* immune responses in this species. Having established a permissive temperature for immune function, this study shows that feral red drum develop substantial humoral responses to a subset of their indigenous bacteria.

MATERIALS AND METHODS

For laboratory-based studies, *S. ocellatus* were spawned from brood stock at SCWMRD, Fort Johnson, S.C. Fingerlings were transported to ponds at the James Waddell Mariculture Center in Bluffton, S.C., where they were reared under standard aquaculture conditions until one to two years old. Animals were then seined from the stock ponds and transported to indoor holding tanks at Fort Johnson. Holding tanks were equipped with a recirculating drip-filtration seawater system and supplied with settled water from Charleston Harbor which was adjusted to and maintained at 25±1°C and 15-20 ppt seawater. Animals were fed squid daily. All fish were equilibrated to the holding tanks for a minimum of 30 day prior to blood sampling.

For field studies, one, two and three year old *S. ocellatus* were collected by stop net on May 10 and June 17, 1993, from the Grice Cove area in Charleston Harbor. Bacterial culture swabs were taken in the field from the mucosal slime on the flanks of captured fish and inoculated immediately onto Trypticase Soy Agar plates, which were incubated at 25°C for 24 - 72 hr. Captured animals were brought to a nearby dock area where visual health assessments, physical measurements and blood samples were taken.

Bacterial isolates were tested and classified by standard methods (MacFaddin, 1980; Austin and Austin, 1987). For use as target antigens in ELISA, all isolates were cultured in Trypticase Soy Broth to mid-log phase, then washed in phosphate-buffered saline (PBS), resuspended to approximately 5 x 10^8 cells/ml and heat killed in a boiling water bath for 30 min. The antigen preparations were aliquoted and frozen at -20°C until use.

Blood samples were drawn from the caudal vessel by syringe and injected into lithium heparin Vacutainer tubes. When appropriate, PBL were isolated over a Ficoll Hypaque gradient. Proliferation assays with isolated PBL were performed by standard procedures using tritiated thymidine (Faulmann *et al.*, 1983).

For serum collections, blood samples were left on ice for at least 30 min, centrifuged, and the resulting serum aliquoted for storage at -20°C. ELISAs with red drum serum employed a mouse monoclonal antibody to the heavy chain of red drum IgM, (RDG013) which was developed in our laboratory. Binding of RDG013 was detected with a commercially available peroxidase-conjugated goat anti-mouse polyclonal antibody (Sigma).

RESULTS

Temperature sensitivity of the *in vivo* humoral immune response in the red drum.

One to two-year old maricultured animals were immunized intraperitoneally with three 50 µg doses of human serum albumin conjugated to dinitrophenyl (HSA-DNP) and emulsified 1:1 with complete (initial immunization) or incomplete (second and third doses) Freund's adjuvant. Animals were bled and boosted on days 1, 11, and 29, and bled only on days 39 and 69. Animals acclimated and held at 25°C developed strong humoral responses to HSA-DNP by day 29 (Figure 2). Titers peaked on day 39 and remained unchanged for at least one month, to day 69 (data not shown). Under the same schedule of immunizations, animals held at 12±1°C failed to develop titers to HSA-DNP above their preimmune levels. Given the range of seasonal temperature fluctuations in the Charleston Harbor, during the winter months (December - February) red drum are exposed to conditions which are non-permissive for primary humoral responses to some types of antigens.

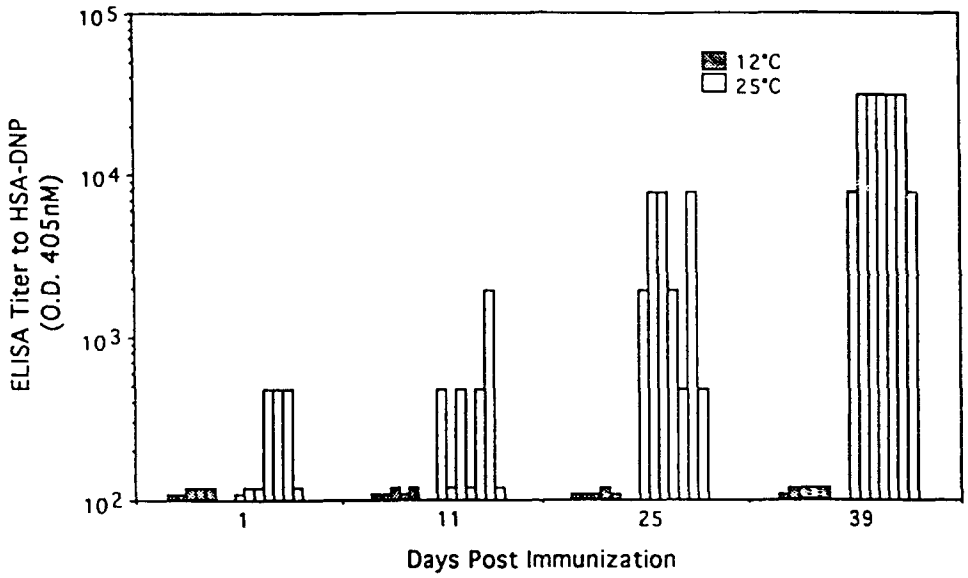

Figure 2. Temperature sensitivity of the *in vivo* humoral response to HSA-DNP. Animals acclimated to 12 or 25°C were immunized with HSA-DNP and humoral antibody responses monitored by ELISA over 39 days (see text for details). The reciprocal endpoint titer against the immunizing antigen is plotted for each animal serum.

Temperature sensitivity of *in vitro* PBL responses to T and B cell mitogens.

In addition to seasonal temperature fluctuations, estuarine fish are exposed to daily temperature changes as a function of the tidal cycle. Clem *et al.* (1984) demonstrated that *in vitro* cellular immune functions in the channel catfish were extremely sensitive to such rapid, short term temperature changes. Using a similar experimental design, we analyzed the sensitivity of mitogen-dependent proliferation in red drum PBL to rapid temperature shifts. PBL were isolated from 25°C acclimated animals. Leukocyte preparations from individual animals were divided in half and immediately placed in parallel cultures at 25°C and a "shifted" temperature, 10, 15, 20, or 30°C. For these studies cells were stimulated with ConA and LPS. The phorbol ester 12-O-tetradecanoyl 13-acetate (TPA, Sigma, St.Louis, MO) was employed as a mitogen positive control. TPA directly

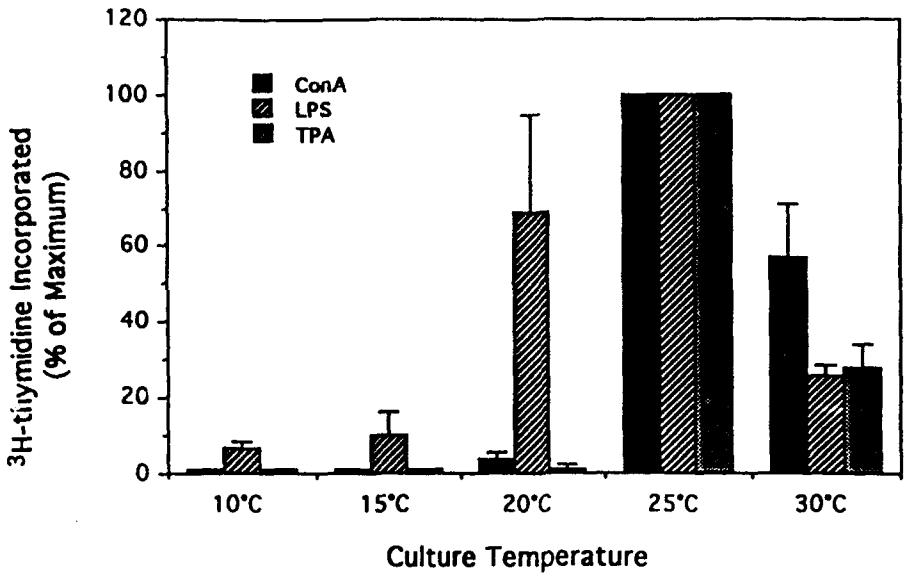

Figure 3. Temperature sensitivity of the *in vitro* humoral response to rapid, short-term temperature changes. PBL were isolated from red drum acclimated to 25°C. Cell preparations from each animal were divided and cultured in parallel at 25°C and one experimental temperature. All cell preparations were stimulated with the indicated mitogen for two days, labeled with ^{3}H-thymidine, and harvested 18 hr later. For each PBL preparation proliferation in response to all mitogens was optimal at 25°C and this value was set at 100%. Proliferative responses for the experimental temperature were expressed as a percentage of the 25°C response. Mitogen concentrations were 50 µg/ml ConA, 100 µg/ml LPS and 20 ng/ml TPA. Six animals were used for each temperature comparison, with triplicate determinations for each tested mitogen.

stimulates protein kinase C, bypassing temperature-dependent changes in membrane rigidity (Truneh *et al.*, 1985). Therefore, temperature-dependent changes in TPA-induced proliferation should reflect general metabolic slowdown. Incorporation of tritiated thymidine was measured after two days of culture. Proliferation was greatest at 25°C, and other values were normalized to the 25°C response (Figure 3). PBL responses to ConA and TPA were dramatically reduced by a shift to 20°C, while a substantial response to LPS remained. However, a shift to 15°C eliminated approximately 90% of LPS response as well. Thus, mitogen-dependent proliferative responses of red drum PBL were extremely cold-sensitive, and, as documented for catfish PBL (Clem *et al.*, 1984), proliferation in response to the classical T cell mitogen ConA was more cold-sensitive than the response to the B cell mitogen LPS. Unexpectedly, responses to TPA mimicked the more cold-sensitive T cell mitogen response, a phenomenon which is currently under study in our laboratory.

In vivo humoral responses to indigenous bacteria at permissive temperatures.

To assess the potential of the red drum as a sentinel species, it was necessary to establish an appropriate biomarker assay, in this case, a rapid and reliable assay for immunocompetence in individual animals. Using an approach similar to that of Robohm *et al.* (1979) bacteria associated with the mucosal slime of animals from a single feral population were cultured, isolated and

Table 1
Profile of anti-bacterial serum reactivities of all red drum collected from Grice Cove on May 19 and June 10, 1993.

Fish Tag No.	t12	t13	t14	t15	t16	t17	t18	t19
865	●		●	●	●	●	●	●
964			●	●	●	●	●	●
965						●		
144			●		●	●	●	●
417								
765								
199								
645	●				●	●	●	●
966					●	●		
618						●		
619				●				
620	●				●	●	●	●
621								
719				●	●			
759					●	●	●	●
940					●	●		
385					●	●		
000						●		

Sera were diluted 1:30 with PBS and reacted in an ELISA against bacterial isolates taken from Grice Cove on May 19, 1993. Each serum was assayed in duplicate and an average taken of the resulting OD (405nm). Background OD values of the secondary (mouse anti-red drum Ig monoclonal antibody) and tertiary (HRP-conjugated goat anti-mouse antibody) reagents on each bacterial isolate were subtracted. Resulting values above 1.2 OD are indicated by ●.

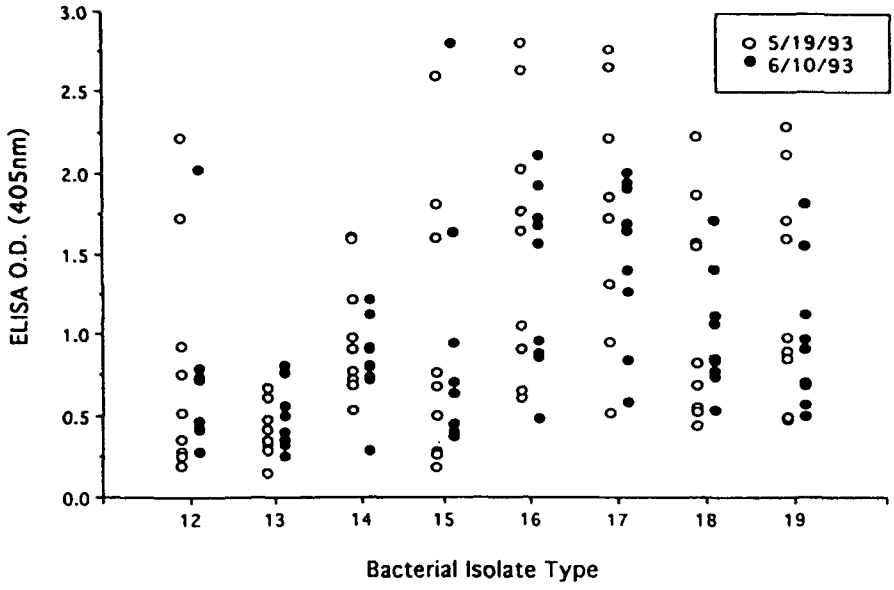

Figure 4. Serum reactivities of red drum from Charleston Harbor against indigeneous bacteria. Sera from healthy fish captured on May 19, 1993, and June 10, 1993, were diluted 1:30 in PBS and reacted in ELISA against bacterial isolates obtained on 5/19/93 from animals in the same population. Reactivity of each serum against each isolate (t12 - t19) is expressed as O.D.(405 nm).

identified. These isolates were used as antigen targets in ELISA to monitor the serum responses of animals from the same red drum population to indigenous bacteria. This approach was taken to minimize problems arising from seasonal changes in bacterial flora and temporal fluctuations in antibody titers.

For these studies, one to three-year old red drum were captured by stop net in the Grice Cove of Charleston Harbor during May and June, 1993, when low tide water temperatures were 23 and 28°C, respectively. All 71 captured red drum were assessed for overt signs of disease or physical lesions, and 63 animals were classified as "apparently healthy". The remaining 8 "minimally healthy" animals exhibited some minor external cuts or parasites. Sera were taken from 18 apparently healthy animals chosen at random for assessment of serum anti-bacterial reacitivities. Bacteria were cultured from the mucosal slime of three animals from each health category captured during the May sampling. Eight discrete colony types were isolated and identified as follows, with the culture number provided in parentheses: *Pasteurella piscidia* (t12), *Enterobacter spp.* (t13), *Flavobacterium spp.* (t14), *Edwardsiella spp.* (t15), *Aeromonas hydrophila* (t16) and *Aeromonas spp.* (t17, t18, t19). There was no apparent correlation between health status and the incidence of each colony type.

When indigenous bacterial isolates were used as antigen targets in ELISA (Figure 4), it was clear that serum reactivities against t13, *Enterobacter spp.*, were poor in all animals tested. In contrast, based on a positive cut-off value of 1.2 OD(405 nm), 12 of 18 animals had strong serum responses to t17, *Aeromonas spp.* However, individual sera exhibited distinctive reactivity profiles. Table 1 shows that 14/18 animals (78%) of tested animals displayed a strong serum response to at least one bacterial isolate.Should future studies identify a similar pattern of serum responses, a streamlined

screening panel composed of t15 (*Edwardsiella spp.*) and t17 (*Aeromonas spp.*) might be suitable for developing rapid, field-based assays for humoral immune function in the red drum.

CONCLUSIONS

The results of these experiments with the red drum have suggested that at environmental temperatures permissive to *in vivo* humoral immune responses, individual animals exhibit distinctive serum anti-bacterial reactivities. This observation confirms previous work by Robohm *et al.* (1979) in populations of winter and summer flounder. However, in the immunological biomarker assay used for the red drum, prevalent indigenous bacteria were isolated concurrently with drawing serum samples for testing humoral responses in the same feral population. This approach minimized the problems introduced by changing bacterial serotypes in the environment, as well as the relatively short duration of humoral immune responses in teleost fish. Within the assessment described here, 78% of feral animals had strong serum responses to *Edwardsiella* and/or *Aeromonas spp.* If similar patterns arise in future sampling, it may be possible to select limited panels of bacterial antigen targets for rapidly assessing immunological health in wild populations of red drum.

In association with scientists at SCWMRD, our laboratory is undertaking a one year study to monitor seasonal changes in serum anti-bacterial responses in red drum from two populations within Charleston Harbor and a third population in Winyah Bay, S.C. In the latter estuary, SCWMRD scientists have observed external evidence of bacterial infections on some red drum during the winter. In addition, water quality and ecological profiles of both estuaries have been initiated recently under the Environmental Monitoring and Assessment Program (EMAP) sponsored by EPA and NOAA. As part of such a multifaceted environmental assessment program, the proposed biomarker assay for monitoring teleost immunocompetence and the use of the red drum as a sentinel species should receive useful scrutiny.

Acknowledgements:

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Chapter 17

Lazarite Alters Copper-Induced *in vivo* Priming of Goldfish Macrophages.

Ann G. Muhvich[1], Andrew S. Kane[1], Thomas Hopkins[2] and Renate Reimschuessel[1]

[1]Department of Pathology,
University of Maryland School of Medicine
10 Pine Street
Baltimore, MD 21201

[2]Biometrics, Inc.
21510 Clarksburg Rd
Boyds, MD 20841

ABSTRACT

Aquatic organisms are sensitive to toxicants in aquatic environments, and may be used as sentinels to determine pollutant exposure. Leukocytes can be used as immune system indicators of heavy metal contamination. Sublethal copper sulfate (100 ppb) exposure of goldfish (*Carassius auratus*) has previously been shown to modify the activation responses of macrophages. Goldfish were exposed to water with or without lazarite, a proprietary compound used to restore natural qualities to the water, and copper sulfate (175 ppb). Pronephric cells isolated from fish exposed to 1)copper, 2)copper-Lazarite, 3)Lazarite and 4)control were stimulated with zymosan, and the chemiluminescence (CL) response was measured. The peak CL response in copper-exposed fish occurred temporally prior to controls, suggesting *in vivo* priming. There was no significant difference between control and Lazarite-exposed fish in response time. However, the copper-Lazarite exposed fish exhibited a significantly longer CL response time than copper exposed fish, which resembled controls. We conclude that *in vivo* use of Lazarite, along with a heavy metal such as copper, can induce a CL response of macrophages from goldfish similar to controls.

INTRODUCTION

Aquatic organisms are subject to changes in water quality. Pollutants, especially at sublethal levels, have been shown to impact aquatic health in particular. The immune system of fish is susceptible to toxicants in the water (Bick, 1982). Some heavy metals, such as copper, have been found to target the hemopoetic organs of fish such as the head kidney, which is analogous to mammalian bone marrow and is the primary lymphoid organ (Elsasser *et al.*, 1986). Therefore, one might expect that cells in these organs, including the macrophages, would be affected by copper exposure.

Phagocytosis and killing of pathogenic microorganisms are functions of macrophages and can be measured *in vitro*. Macrophage function can be correlated with the health of fish, and has been used as a method for comparing the immune function of fish from polluted and clean waters (Warinner *et al.*, (1988).

Fish pronephric leukocytes emit chemiluminescence (CL) when stimulated (Plytycz *et al.*, 1989; Tam and Hinsdell, 1990). This reflects production of reactive oxygen intermediates (ROIs) such as superoxide anion (O_2^-) which are necessary for killing of microbial pathogens. CL has been used to measure ROIs released from phagocytes after exposure to xenobiotics (Stave *et al.*, 1983). Fish cells from polluted rivers have been shown to exhibit depressed peak CL responses (Warinner *et al.*, 1988). The CL response of oyster hemocytes exposed to 4 ppm copper *in vivo* and *in vitro* was examined and was shown to be depressed after *in vivo* exposure. Other metals, such as cadmium, zinc and aluminum showed enhanced CL response after *in vitro* exposure (Larson *et al.*, 1989).

Experiments here were designed to test the immunotoxic effects of sublethal exposures of Lazarite, a proprietary compound, and copper sulfate on goldfish after 96 hr exposure. The CL of head kidney macrophages is the bioindicator used to evaluate the cellular immune response.

METHODS

Animals: 8-15 cm goldfish *Carassius auratus* (Hunting Creek Fisheries, MD) were acclimated for a minimum of 3 weeks in the following laboratory conditions. The goldfish were prophylactically treated with malachite green for three day in static 50 gallon tanks. Subsequently, the goldfish received trout grower diet (Ziegler Bros., PA #38-480) daily and were maintained in recirculating 50 gallon fiberglass tanks having a coarse and biological filtration and an average ambient temperature of 21°C and a 16 hr light:8 hr dark photoperiod. Test animals were not fed for 1 day prior to or during testing.

Chemicals and reagents: The heavy metal used was cupric sulfate ($CuSO_4$) (Baker Chemicals, NJ) and was dissolved in distilled water and added to 50 gallon tanks at a concentration of 175ppb. Lazarite, a proprietary compound was used at concentrations adequate to keep minerals at natural levels. Lazarite was mixed in a 150 gallon tank and titrated with HCl to pH 7.9 before adding to 50 gallon polypropylene exposure tanks.

Toxicity testing: Stock $CuSO_4$ (175 ppb) and Lazarite were added to 161 L containing freshly dechlorinated tap water and mixed well. Five fish were added to each of four static tanks containing either copper, copper-Lazarite, Lazarite or control water with no aeration or filtration, and exposed for 96 hr. This treatment was repeated 5 times.

Tissue collection: Goldfish were sacrificed by decapitation, severing just caudal to the operculum. Head kidneys were removed using sterile forceps and placed in sterile 15 mL glass tissue grinders, hand homogenized using approximately 25 strokes in 5 mL wHBSS (HBSS without phenol red + heat inactivated 10% fetal bovine serum + antibiotics (Penecillin/Streptomycin at a concentration of 10 mL/L)), and removed to 15 mL polypropylene sterile centrifuge tubes. Gills were removed and placed in 10% neutral buffered formalin, paraffin fixed and stained with Hematoxylin and Eosin after sectioning at 6μ.

Cell collection: Cells were centrifuged on a 3 ml Histopaque gradient (d=1.077g/cm^3-Sigma Chemicals) at 1500 x g for 20 min at 10°C, buffy coat removed and wash twice in wHBSS (5 min at 1000 x g). Cells were counted twice on a hemocytometer, and viability determined to be greater than 96% using the trypan blue exclusion test. Cells were resuspended in wHBSS at a concentration of 5 x 10^5 cells/mL..

Chemiluminescence Assay: The oxidative burst which is produced by stimulated macrophages was quantified using a Beckmann LS-150 ambient temperature scintillation counter in the out-of-coincidence mode with a single photon monitor. Luminol (Sigma, St.Louis, MO.) was used to amplify the CL response of phagocytic cells, and was prepared according to Scott and Klesius (0.78g KOH, 0.618 g boric acid, 0.014 g luminol in 10 mL distilled water (stored for 3 day max)). A stock A-solution was diluted 1:1000 in Hanks Balanced Salt Solution for use (working solution). Each vial contained 0.5 mL luminol, 0.5 mL cells in wHBSS, and 0.5 mL zymosan as stimulus (1 mg/mL in wHBSS), and vials were measured in duplicate. Blank vials contained cells and luminol, but no zymosan. Basal CL was maintained at 3-5 x 10^4 cells/mL. Vials were immediately placed in the counter and measurements made approximately 4 min apart for 90 min, or until a significant decline in peak CL occurred. All solutions and materials were kept in the dark as much as possible.

Gill Histopathology: Gills were evaluated by grading for hyperplasia on a scale of 0-4 with 0 representing no hyperplasia and 4 representing complete fusion of secondary epithelia.

Experimental Design (Figure 1)

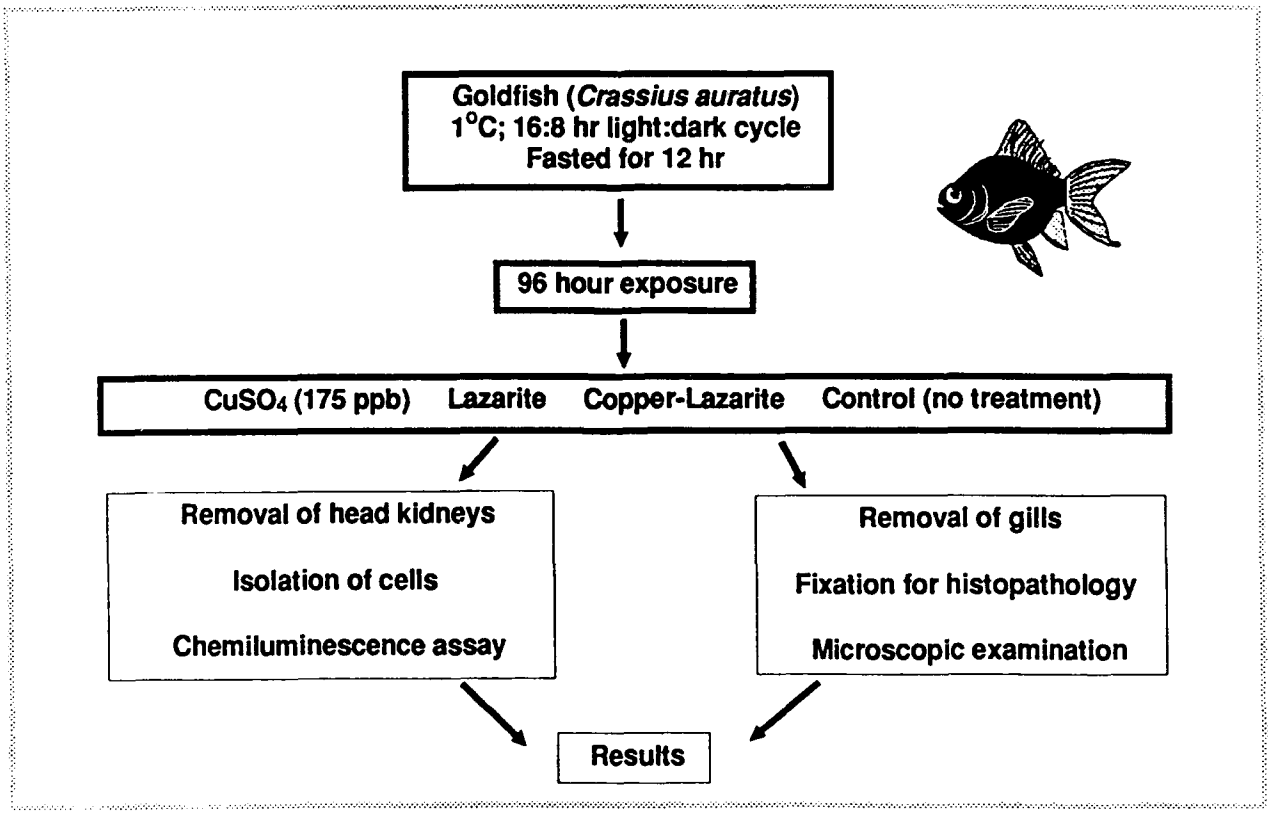

Figure 1. Experimental design

RESULTS

The effect of copper exposure alone on macrophage CL is shown in Figure 2 (a representative experiment). In copper exposed goldfish, lag time before a peak response was shorter and CL profiles demonstrated an increased slope compared to controls. In an analysis of 35 fish, peak CL was achieved in 59 min for controls and 24 min for copper treated samples (See Table 1).

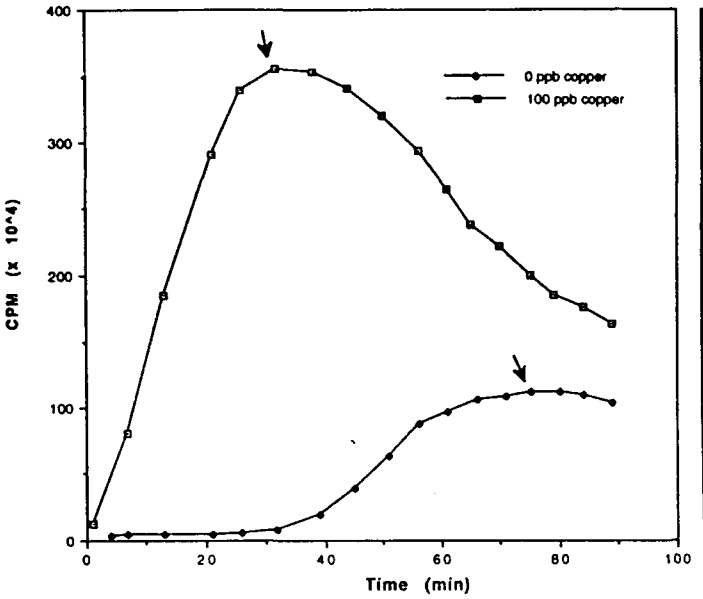

Figure 2. Chemiluminescent response from 96 hr copper-exposed goldfish (100 ppb). Results from a representative experiment (arrows indicate peak response)

Table 1
Peak response time for CL response in treated and control goldfish

Treatment	Peak response time in min mean ± standard error	number of fish
Control	58.6(5.6)*	21
Copper	23.9(5.5)*,++	14
Copper-Lazarite	37.9(4.0)++	24
Lazarite	54.0(3.6)	20

*Significantly different at $p < 0.01$

++Significantly different at $p < 0.05$

The mean times to peak CL response of Lazarite and copper- exposed goldfish were not significantly different from controls (see Table 1). However the mean time to peak CL response of copper-Lazarite treated fish was significantly longer than copper treated fish.

Copper induces hyperplastic gill epithelium, while Lazarite reduces the degree of hyperplasia. Control gills (Figure 3A) demonstrate distinct, regular secondary lamellae. *In vivo* copper exposure results in occlusion of secondary lamellar epithelium (Figure 3B), decreasing exposed respiratory epithelium. Copper-Lazarite exposure demonstrates mixed results with some fused and some singular lamellae (Figure 3C).

Figure 3. Histopathology of *C. auratus* gills (Hematoxalin and Eosin stain). **A.** Control (magnification = x400), **B.** 96 hr exposure to 175 ppb copper sulfate(magnification = X200), **C.** 96 hr exposure to copper sulfate and lazarite (magnification = X200).

DISCUSSION

The events which cause macrophages to produce CL or emit a respiratory burst are dependent on production of reactive oxygen intermediates (ROIs) and have been studied in mammals and fish. A cascade of events initiated by an external stimulus such as zymosan activates protein kinase C (PKC), which in turn activates the membrane enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In the presence of molecular oxygen, NADPH oxidase produces O_2^-. Luminol reacts with oxygen intermediates such as O_2^- and H_2O_2 produced by dismutation of O_2^- to yield a quantity of light (Trush *et al.*, 1978).

The data presented in this paper demonstrate that *in vivo* copper exposure of 175 ppb can cause a decrease in response time to peak CL in goldfish, suggesting *in vivo* priming. Addition of Lazarite (a proprietary compound) to copper exposed tanks can prolong the CL response time in phagocytes. The CL kinetics of copper-Lazarite exposed fish resemble controls.

Similar results were found in human patients with cystic fibrosis (Graft *et al.*, 1982). Granulocytes from patients with cystic fibrosis have been shown to exhibit decreased CL response time compared to controls. Granulocytes from patients with airway disease and more severe cystic fibrosis have a more rapid onset of the CL response. The authors suggested that these cells appeared 'primed'. Cystic fibrosis patients develop recurrent pulmonary infections, characterized by distended bronchioles from thick mucus, and transformation of the columnar cell lining into squamous epithelium. It has been proposed that hypersecretion of calcium by mucous glands is a primary defect of this disease.

Our laboratory examination of histological sections of copper exposed goldfish gills shows mucous cells, but not a proliferation of mucous cells as the literature suggests (Reimscheussel *et al.*, 1991).

A possible mechanism for the *in vivo* priming response seen here may be via increased intracellular calcium levels. *In vitro* studies in oyster toadfish macrophages have demonstrated that tributyltin (TBT) exposure activates macrophages (Rice and Weeks, 1989). Results suggested that TBT causes a calcium influx and a resultant enhancement of CL response.

Lazarite has been shown here to ameliorate the response of *in vitro* copper exposure as measured by chemiluminescent assay. The clinical significance of a decrease in response time of head kidney macrophages to a stimulus is not understood. Decreased response time of head kidney macrophages may indicate a hyperactive immune response. Lazarite may have some beneficial properties in controlling this response.

In vivo, chemicals may exert their immune modulation indirectly or directly. It is possible that pronephric cells of copper treated golfish are primed as a result of active proliferation associated with this treatment. However, no correlation was seen between degree of hyperplasia in the gill epithelium and peak CL kinetics. Metabolic activation of macrophages may determine a compound's ultimate effect.

ACKNOWLEDGMENT

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Chapter 18

Ultraviolet-B Radiation and the Immune Response of Rainbow Trout

David L. Fabacher[1], Edward E. Little[1], Susan B. Jones[1], Edward C. De Fabo[2], and Lindsay Johnson Webber[2]

[1]National Biological Survey
National Fisheries Contaminant Research Center
4200 New Haven Road
Columbia, Missouri 65201

[2]The George Washington University Medical Center
Department of Dermatology
2300 I St. N.W.
Washington, D.C. 20037

ABSTRACT

As part of a larger study on global climate change and ozone depletion we are investigating the effects of ultraviolet-B (UVB) radiation on fishes. We conducted a number of experiments to explore the possible effects of UVB radiation on the immune response of juvenile rainbow trout *Oncorhynchus mykiss*. In one study, the fish developed sunburn and fungal infection on the dorsal skin after exposure to levels of UVB that simulated ambient solar UVB levels observed at mid-latitudes. In a separate study, UVB-exposed rainbow trout with surgically administered dorsal lesions developed fungal infection on the lesions and surrounding skin. Many of these fish subsequently died within a 9 day exposure period. Fish with surgical lesions, but not exposed to UVB radiation, did not develop fungal infection and did not die. In mammals, UVB-induced immunosuppression is thought to occur through the isomerization of urocanic acid or the formation of DNA pyrimidine dimers, or through some interaction between the two. We found a substance that appeared, upon HPLC detection, to be trans-urocanic acid in the skin of UVB-exposed and unexposed rainbow trout. Neurotransmitter stimulation of adrenoceptors may be involved in changes in pigmentation observed in UVB-exposed fishes. We measured adrenoceptors in skin membranes from rainbow trout exposed to UVB and found a decrease in α_2-adrenoceptors compared with fish not exposed to UVB. Results of our study indicate that prolonged exposure of juvenile rainbow trout to mid-latitude levels of solar UVB may play an important role in the initiation of certain disease outbreaks and may decrease survival of fish that have lesions on the dorsal skin.

INTRODUCTION

Depletion of the earth's protective ozone layer could lead to increased levels of UVB radiation (290-320 nm) at the earth's surface, with subsequent adverse effects on terrestrial and aquatic animals (Gleason and Wellington, 1993; Hader, 1993; Kerr and McElroy, 1993;.van der Leun and de Gruijl, 1993)

UVB can penetrate the water column and can have a variety of effects on fishes, including sunburn, malformations and mortality (Bullock, 1982; Smith *et al.*, 1992). When Hinrichs (1925) exposed early cleavage stages of *Fundulus heteroclitus* embryos to ultraviolet radiation, the resultant larvae had severe malformations, including inadequate circulation and abnormal tails. Bell and Hoar (1950) irradiated eggs and alevins of sockeye salmon *Oncorhynchus nerka* with ultraviolet radiation. Histological examination of the alevins revealed changes in the epidermis and fibroelastic layers of irradiated regions of the skin. Dunbar (1959) observed skin lesions on rainbow trout fingerlings exposed to direct sunlight in outdoor pools and to a sunlamp in the laboratory. Fish exposed in pools and in the laboratory developed identical necrotic areas around the dorsal fin and behind the head. In both cases there was increased mortality compared with unexposed fish. Dunbar (1959) also observed darkening of the dorsal areas of the skin of fingerling rainbow trout exposed to a sunlamp. Darkening of the skin of fishes could, at least in part, result from neurotransmitter stimulation of α-adrenergic or β-adrenergic receptors (adrenoceptors) (Schliwa, 1984). Bullock and Roberts (1981) described skin lesions in rainbow trout exposed to abnormally high sunlight levels while held in outdoor tanks.

The immune response in fishes can be depressed by a wide range of environmental stress factors, including chemical contaminants, drugs and radiation, such as X-radiation and gamma-radiation (Zeeman and Brindley, 1981; Anderson *et al.*, 1984). Once the immune system has been depressed, fishes may be more susceptible to infection by pathogens (Sindermann, 1979; Weeks *et al.*, 1986; Anderson, 1990).

Urocanic acid has been suggested as a mediator of UVB- induced immunosuppression in mammals by isomerizing from the trans to the cis form following the absorption of UVB photons (DeFabo and Noonan, 1983; De Fabo *et al.*, 1990; Noonan and De Fabo, 1992). The cis-urocanic acid may then bind to receptors to modulate cell-mediated immunity (Norval *et al.*, 1990; Palaszynski *et al.*, 1992). Urocanic acid occurs in the skin of mammals predominantly as the trans-isomer. With UVB irradiation, isomerization of the trans-isomer of urocanic acid to the cis-isomer occurs along with suppression of the immune response. The evolutionary significance of this mechanism in mammals is thought to be protective, to prevent an uncontrolled autoimmune attack on sun-damaged skin cells (Noonan and De Fabo,1992). Exposure to high levels of UVB resulting from stratospheric ozone depletion could lead to overstimulation of this mechanism causing an increased susceptibility to pathogens. A similar mechanism of immunosuppression may occur in fishes.

In a preliminary observation in our laboratory, rainbow trout embryos exposed to UVB radiation developed fungal infection. This observation prompted us to determine if UVB-exposed juvenile rainbow trout were more susceptible to fungal infection than unexposed fish. We also wanted to determine if urocanic acid occurred in the skin of rainbow trout and if there was a change in adrenoceptors in UVB-exposed fish.

MATERIALS AND METHODS

Solar Simulator

The solar simulator was a 0.61 m wide by 1.83 m long light fixture containing ten 160-watt cool white lamps (General Electric Co., East Cleveland, OH), four 160-watt UVB313 Lamps (National Biological Corp., Twinsburg, OH), eight 160-watt UVA365 lamps (National Biological Corp., Twinsburg, OH), two 20-watt cool white lamps (Osram Sylvania, Danvers, MA), two 20-watt SF20 sun lamps (Philips Lighting Co., Somerset, NJ), and eight 75-watt halogen incandescent flood lamps (General Electric Co., East Cleveland, OH). The simulator was suspended over a water bath of similar dimensions and was enclosed with reflective specular aluminum. The UVB lamps were controlled by a recycling 24 hr timer, which turned them on for 5 hr during a simulated solar noon. The cool white and UVA fluorescent lamps were controlled by a second timer to operate for a 16 hr period, simulating a midsummer photoperiod. The UVB exposure was 5 hr per day for 7 days. The testing area was air conditioned because of the heat generated by the simulator, and the water bath was supplied with continuously flowing chilled water, or by recirculating water that was regulated by a thermostatically controlled chiller/heater. The water temperature was 18°C.

The output of the solar simulator was calibrated by measuring the spectral irradiance of the simulator at 15.2 cm intervals along a matrix beneath the lights using an Optronic Laboratories (Orlando, FL) Model 752 spectroradiometer equipped with a 15.2 cm-diameter integrating sphere. The scanning range was 290 to 800 nm at one nm step intervals using a signal integration time of 1040 msec per nm. The spectroradiometer was calibrated with a National Institute of Standards and Technology traceable tungsten lamp over the range of 290 to 800 nm at 1 nm intervals. In addition, the alignment of the holographic gratings in the spectroradiometer was checked daily for wavelength accuracy at 302.2 nm using a fluorescent calibration lamp. The voltage output (gain) at 550 nm gain was also checked daily using a tungsten lamp at 550 nm, and the calibration file was adjusted for reductions or increases in the system response.

Test Organisms and Exposures

Rainbow trout *Oncorhynchus mykiss* were obtained from the Saratoga National Fish Hatchery, WY as eyed embryos and were maintained at the National Fisheries Contaminant Research Center Columbia, MO. Fish were housed in raceways where illumination was provided with about 270 lx of artificial light in a 16:8 hr light to dark photoperiod. Water quality variables were typically as follows: dissolved oxygen, 8.2 mg/L; conductivity, 575 µS/cm; pH, 7.8; water hardness, 341 mg/L as $CaCO_3$; alkalinity, 256 mg/L as $CaCO_3$; and temperature, 18°C. Fish were used in experiments at about 60 days posthatch and had a mean length [SD] of 4.0 cm [0.33] and a mean weight [SD] of 0.53 g [0.14].

Exposures were conducted in open-top-glass air-lift chambers (Cleveland *et al.*, 1991), which were in the water bath under the solar simulator and contained 2 L of water of the same temperature and quality that the fish were maintained in prior to testing. The distance between the solar simulator and the water surface was 61 cm. There were three treatments: control, low, and high. The control conditions (average UVB irradiance of 4.3 $\mu W/cm^2$, range from 4.0 to 4.6 and a dose of 0.08 J/cm^2/day), were created by covering the top and bottom of each chamber with 0.76 mm polycarbonate and then covering the sides with 0.127 mm mylar. A low UVB irradiance was generated by covering the top and bottom of each chamber with cellulose acetate that had been aged by a 72 hr

exposure to UVB, to ensure stable UVB filtration. The decrease in overall illumination due to cellulose acetate filtration was ≤ 5%. In this treatment, cellulose acetate of 0.127 mm thickness created an average low irradiance of 190 $\mu W/cm^2$ (173 to 209 $\mu W/cm^2$) and a dose of 3.42 J/cm^2/day. The highest UVB irradiance of 357 $\mu W/cm^2$ (320-400 $\mu W/cm^2$) and a dose of 6.43 J/cm^2/day was a direct, unfiltered exposure.

In the first of four experiments, three replicate groups of 15 x 15 x 23 cm air-lift chambers per treatment were randomly distributed under the solar simulator. Prior to positioning of each chamber, a spectroradiometric scan of UVB at the water surface from 290 to 800 nm at 1 nm intervals was made at each chamber location using the filter material applied at that location. Each chamber was then placed in its position. The bubbling action of an air stone in each chamber created a water flow of about 0.6 L/min entering a screened area at the bottom of one side of the chamber and draining at the top of the partially screened partitioned corner of the chamber confining the air stone. Disturbance of the water surface by the bubbling action was minimal and restricted to one corner of the chamber. Five fish were stocked in each chamber. Control, low, and high dose treatment groups were exposed for 7 days, and the entire experiment was repeated (total of 18 observations). The fish were fed 24 hr old *Artemia* sp. and salmon starter several hr prior to and at the conclusion of the UV photoperiod each day. The chambers were cleared of food and debris to maintain a high level of optical clarity. Fish were observed daily for the presence of sunburn and fungal infection.

In the second experiment, fish were anesthetized in a solution of tricaine methanesulfonate, the dorsal part of the caudal fin was surgically removed from each fish with a curved microscissors. Fish were then exposed for 9 days to 0.08 (control: 15 fish) or 6.43 J/cm^2/day (high dose: 17 fish) UVB as described previously. The experiment was repeated with 10 fish per group and with only the dorsal fin removed from each fish.

In the third experiment, fish were exposed to the control and high treatments for 9 days for adrenoceptor determinations. There were 4 separate replicates/group, 5 fish/replicate.

In a fourth experiment, 5 fish/control and a high dose group were exposed for 4 days for urocanic acid determination. Exposure conditions were as previously described.

Treatment effect on fish response was evaluated using analysis of variance techniques performed with the Statistical Analysis System (SAS 1989). Cumulative percentages of fish response (sunburn and fungus) were arcsine transformed and analyzed using a randomized complete block model to account for effects between experimental trials (Snedecor and Cochran 1980). Fish from control groups did not develop sunburn or fungus (mean response = zero) and these observations were omitted from the analysis. Fisher's LSD test ($p<0.05$) was used to determine differences between treatment means and if treatment means were significantly different from zero.

Removal and storage of skin from fish

Fish were euthanized in a container of iced water for 5 min, the water decanted off, and the container placed into a -20°C freezer for 1 min. This made the skin easier to remove because the fish was rigid. Using a binocular dissecting microscope and pointed forceps, a fish was held carefully on a watch glass while the skin posterior to the head and opercula was punctured and gently teased and pulled from the fish. Whole fish skins were used in the assays for adrenoceptors because a large sample was needed. For the determination of urocanic acid, only the dorsal portion of the skin was

used. Each skin sample was weighed, placed in an Eppendorf tube, and used immediately or fast-frozen on dry ice. Fast-frozen fish skins were stored at -80°C.

Adrenoceptors

Freshly removed whole skins from 5 fingerling rainbow trout were pooled and minced with scissors. The tissue was homogenized in 20 mL of 50 mM Tris pH 8.0 buffer with a Tekmar Tissumizer (Model SDT) for 20 sec at high speed followed by filtration through 53 μm nylon mesh. The suspension was centrifuged at 49,000 x g for 15 min. The pellet was resuspended in 20 mL of the same buffer, homogenized and centrifuged as before. The final pellets were resuspended in 25 mM glycylglycine, pH 7.6 for α_2-adrenoceptor binding or 50 mM Tris, pH 8.0 for β-adrenoceptor binding. Tissue concentration was adjusted to yield approximately 0.5 mg/mL protein. Protein concentration was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard. Radioligands were purchased from Dupont-New England Nuclear (Boston, MA) and were stored at -20°C. The binding assay for adrenoceptors was performed as previously described (Bylund *et al.*, 1982). Briefly, 970 μL of tissue homogenate were incubated with 20 μL[^{3}H] yohimbine (YOH; α_2-adrenoceptor selective) or [^{3}H] dihydroalprenoloi (DHA; β-adrenoceptor selective) for 45 min to measure total binding. Non-specific binding was defined by radioligand binding in the presence of 100 μM norepinephrine or 1 μM propranolol for α-adrenoceptors and β-adrenoceptors respectively. Specific binding was determined by calculating the difference between total and non-specific binding. Incubations were terminated with rapid filtration through Whatman GF/B glass fiber filters. The filters were rinsed with 12 mL of ice-cold 50 mM Tris-HCl (pH 8.0 at 25°C) and transferred to vials containing 10 mL scintillation cocktail and their radioactivity determined by scintillation spectroscopy at an efficiency of 46%. This experiment was repeated four times, and the data were analyzed with a paired t-test (Graphpad InStat program, San Diego, CA).

Urocanic acid

Isomers of urocanic acid were determined with High Performance Liquid Chromatography (HPLC). Frozen fish skins were thawed and dorsal skin (approximately 10 mg) used for urocanic acid determination. The skin was minced into small pieces and 100 μL of 1 M potassium hydroxide (KOH) was added to each skin sample. The sample was vortexed and placed on ice for 10 min. and then centrifuged for 5 min in a microcentrifuge. The supernatant was then pipetted into a clean extract tube. The wash process was repeated twice; once with 100 μL of 1 M KOH and once with 200 μL HPLC grade water. The supernatants from these washes were pooled with the original supernatant. Then, 200 μL of 0.67 M phosphoric acid was added to the extract tube containing the pooled supernatant, vortexed, and centrifuged for 5 min at maximum speed in the microcentrifuge. Due to the excessive viscosity of the extract, further processing was required to improve the quality of the sample and to protect the HPLC column. The phosphoric acid supernatant was removed and 100 μL of 1 M KOH added. The supernatant was then vortexed and centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant was removed, and 100 μL of 0.67 M phosphoric acid were added. This mixture was vortexed and centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant was then assayed for trans- and cis-urocanic acid using HPLC as described by Reilly and De Fabo (1991).

RESULTS

Juvenile rainbow trout exposed to levels of UVB radiation comparable to ambient solar levels observed at mid-latitudes developed sunburn (Figure1). The presence of sunburn was a qualitative observation. Sunburn appeared as a darkening of the skin on the dorsal surface of the fish. A decrease in mucus was apparent on the darkened areas of the fish. Sunburn generally occurred in the skin of most fish just posterior to the head and anterior to the caudal fin and ventrally to about the lateral lines. The largest area of sunburn usually occurred just posterior to the head and anterior to and around the dorsal fin. The incidence of sunburn, initially observed on the third day of exposure, increased dramatically over time among fish in both treatment groups (Figure 1). Fish from both high and low treatment groups exhibited a significant incidence of sunburn by the third day of exposure (high treatment, P=0.002; low treatment, P=0.006). The level of response between high and low groups was not significantly different on any day. Sunburn was not observed among control fish.

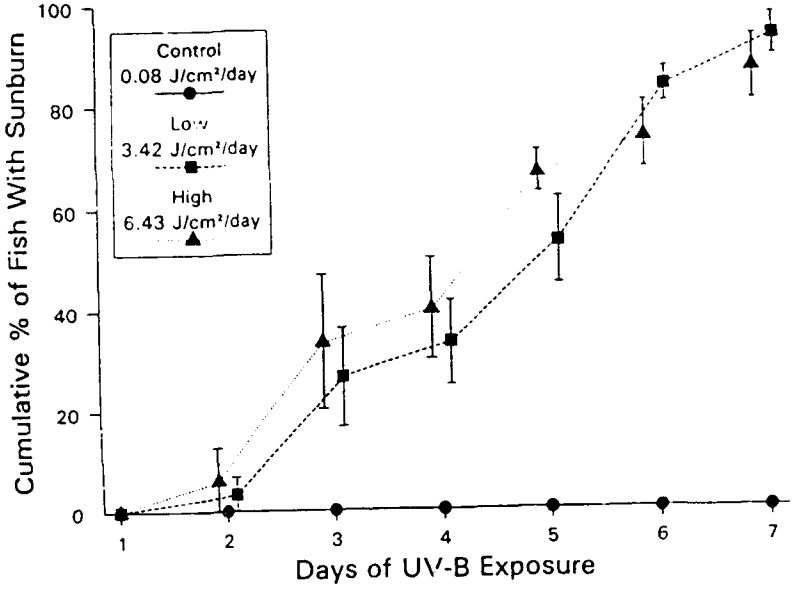

Figure 1. Percent (±S.E.) of UVB-exposed rainbow trout with sunburn

Fish exposed to the low and high doses of UVB developed fungal infection (Figure 2). Like sunburn, the presence of fungal infection was also a qualitative observation. Fish from both low and high treatment groups exhibited a significant incidence of fungal infection by the sixth day of exposure (high treatment, P=0.022; low treatment, P=0.032). The incidence of fungal infection between high and low treatment groups was not significantly different on any day. Fungal infection was not observed on control fish. Fungal infection appeared as mycelia on the darkened areas and on the dorsal fin. Later, patches of fungus covered the darkened areas and the dorsal fin giving those areas a white to gray colored appearance. Fungal patches were isolated from dorsal fins, cultured with *Saprolegnia parasitica* (American Type Culture Collection, Rockville, Maryland), and observed to be *Saprolegnia* sp. Like the manifestations of sunburn (Figure1), the incidence of fungal infection was about the same for both exposure groups.

Rainbow trout that had their fins surgically removed exhibited sunburn and then developed fungal infection covering most of the skin on the dorsal surface of the fish (Table 1). Many of these fish subsequently died. Fish with their fins removed, but not exposed to high UVB radiation, did not

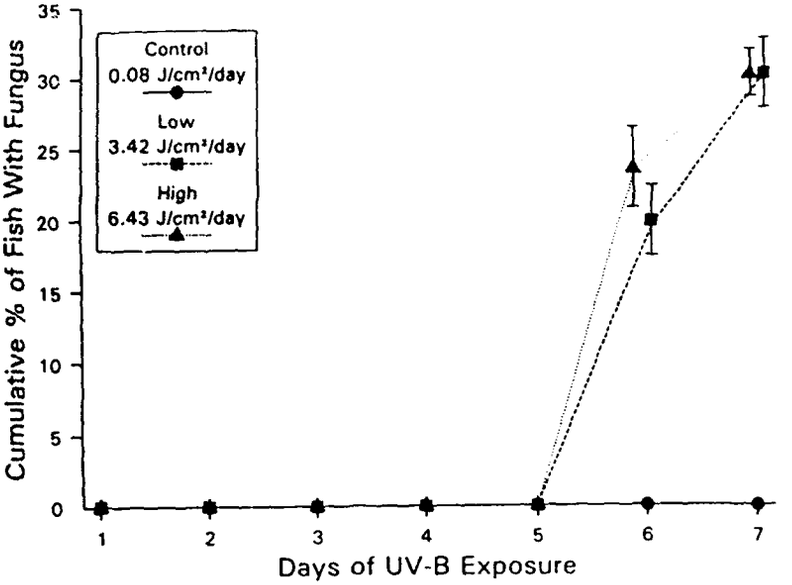

Figure 2. Percent (±SE) of UVB-exposed rainbow trout with fungus

Table 1.
Percent mortality of control and UVB-exposed rainbow trout.

Experiment	Treatment[a]	Number of fish	Mortality (%)
1	Control	15[b]	0.0
	High UVB	17[b]	29.0
2	Control	10[c]	0.0
	High UVB	10[c]	70.0

[a]Fish were exposed to UVB as described for the first experiment but over a 9 day period. UVB exposure: control 0.08 J/cm^2/day; High UVB-6.43 J/cm^2/day.
[b]Dorsal part of the caudal fin removed.
[c]Dorsal fin removed

develop fungal infection and did not die. These results are tentative because the treatments were not replicated.

Adrenoceptor binding in skin membranes of rainbow trout with [^{3}H]DHA (β-adrenoceptor selective) and [^{3}H]YOH (α_2-adrenoceptor selective) are shown in Table 2. In single point experiments (one concentration of radioligand), there was a significant decrease in [^{3}H]YOH binding in membranes from UVB-exposed fish compared with control fish. There was no difference between [^{3}H]DHA binding in skin membranes from control or high-dose UVB exposed fish.

We found a substance that appeared, upon HPLC detection with the appropriate standard, to be trans-urocanic acid in the dorsal skin from UVB-exposed and unexposed rainbow trout (Table 3). None of the cis-isomer was detected.

Table 2.
α_2- and β-Adrenoceptor radioligand binding in skin membranes of rainbow trout from control and high UVB treatments.

Radioligand	fmol/mg protein	
	Control[a]	UVB[a]
[^{3}H]YOH[b]	59 ±18[c]	42 ±17[c,d]
[^{3}H]DHA[b]	20 ± 8[c]	22 ± 8[c]

[a]Fish were exposed to UVB as described in Table 1.
[b] [^{3}H] YOH and [^{3}H] DHA are radioligands specific for α_2-and β-adrenoceptors respectively
[c] Values are mean ±SEM for 4 separate replicates of the experiment, the samples from each replicate contained the skins of 5 fish
[d] Significantly different from control, p=0.002

Table 3.
Urocanic acid in dorsal skin of rainbow trout from control and high UVB treatments

Urocanic acid	ng/mg skin	
	Control[a]	UVB[a]
Trans-isomer	58.2[b]	76.2[b]
Cis-isomer	ND[c]	NDc

[a]Fish were exposed to UVB as described in Table 1, but over a 4 d exposure
[b]Mean values for 5 control and 5 UVB-exposed fish
[c]Not detected at 10 ng/mg tissue

DISCUSSION

Juvenile rainbow trout were exposed to levels of UVB radiation comparable to mid-latitude ambient solar UVB levels. The manifestations of sunburn observed in rainbow trout exposed to UVB included darkening of the dorsal skin and an apparent decrease in mucus on the surface of the dorsal skin. Fingerling rainbow trout exposed to a sunlamp exhibited darkening of the dorsal areas of the skin and with prolonged exposure these areas were rough to the touch, indicating that the mucous cells were damaged (Dunbar, 1959). Bullock and Coutts (1985) described skin damage in rainbow trout exposed to solar ultraviolet radiation while held on a high-altitude fish farm in Bolivia. In a comprehensive treatise on ultraviolet radiation and fishes, Bullock (1988) included a detailed description of the progression of UVB radiation damage in the skin of rainbow trout. Bullock and Roberts (1992) found that UVB radiation compromised wound repair in the skin of Atlantic salmon *Salmo salar*.

In our study, not all fish in the same exposure chamber exhibited darkening of the dorsal skin and subsequent fungal infection. Bullock (1988) also observed considerable variability in response of individual fish to intense simulated solar radiation. Some fish exhibited darkening of the dorsal

skin along the entire dorsal surface of the fish, while others appeared to retain normal coloration. With continued radiation, some of the fish which retained coloration began to show signs of sunburn. Bullock (1988) suggested that the tolerance of some fish to solar radiation may result from elevated levels of some photoprotective factor of genetic origin.

Shechmeister *et al.* (1962) reported that goldfish, *Carassius auratus*, exposed to x-rays and then injected with *Aeromonas salmonicida* died sooner than either nonirradiated, injected controls or irradiated, noninjected fish. Knowles (1992) reported that the humoral immune response was lower in rainbow trout exposed to high levels of gamma-radiation compared with the response of nonirradiated controls or fish given a low dose of exposure. Irradiated fish were also more susceptible to bacterial infection.

The mucus of fishes is involved in resistance to infection (Fletcher, 1978; Ingram, 1980). Bell and Hoar (1950) reported a casual observation of skin injuries (as evidenced by fungal infection and changes in pigmentation) in coho salmon *Oncorhynchus kisutch* fry and goldfish irradiated with ultraviolet radiation from a sun lamp. In our study, the fungal infection may have resulted from UVB-induced degeneration of the epidermis. Epidermal degeneration may have led to less mucus on the surface of the skin, which then allowed the opportunistic fungus to grow.

Rainbow trout exposed to UVB and with a fin removed were very susceptible to fungal infection. Many of these fish died after being observed with substantial fungal infection. The fin removal plus radiation may have enhanced their susceptibility to fungal infection-related mortality; although it is not clear what mechanisms were involved.

Pigmentation of the skin of fishes is under humoral and neural control. One neurotransmitter may regulate both aggregation and dispersion activities of melanophores, depending on stimulation of either α-adrenoceptors or β-adrenoceptors (Schliwa, 1984). When the neurotransmitter concentration is low, β-adrenoceptors are stimulated and melanosome dispersion occurs. At higher neurotransmitter concentrations α-adrenoceptors are stimulated and aggregation of melanosomes occurs. McArdle and Bullock (1987) reported widespread dispersion of melanosomes in the dermis of Atlantic salmon that were thought to have been exposed to excessive solar ultraviolet radiation. Darkening of the dorsal areas of UVB-exposed fishes is probably the result of melanosome dispersion which occurs in response to UV radiation.

Neurotransmitters may be involved in the immune response of fishes. Flory (1990) demonstrated that in cultures of splenic leukocytes from rainbow trout, receptor-mediated *in vitro* induction of antibody cells can be stimulated by autonomic neurotransmitters or their analogs. Thus, autonomic neurotransmitters interacting with cellular receptors may affect immune function in fishes. In our study, it is not clear why there was a decrease in α_2-adrenoceptors in skin membranes from UVB-exposed rainbow trout. The decrease in α_2-adrenoceptors may have been related to the skin darkening observed on the dorsal skin of sunburned fish.

There is concern that elevated UVB radiation as a result of ozone depletion may cause immunosuppression in terrestrial mammals (Fisher and Kripke, 1977, 1982; De Fabo and Kripke, 1979, 1980; De Fabo *et al.*, 1990). In mammals, in separate studies, a compromised immune response has been associated with the formation of pyrimidine dimers and the isomerization of urocanic acid (De Fabo and Noonan, 1983; Applegate *et al.*, 1989; Kripke *et al.*, 1992; Noonan and De Fabo, 1992). Similar mechanisms of immunosuppression may occur in fishes. Ultraviolet radiation-induced pyrimidine

dimers have been measured in fathead minnow *Pimephales promelas* embryos and in the skin of platyfish of the genus *Xiphophorus* (Applegate and Ley, 1988; Ahmed and Setlow, 1993).

We measured a substance with HPLC that appeared to be trans-urocanic acid in the skin of UVB exposed and unexposed rainbow trout, but we did not detect cis-urocanic acid. Kawai and Sakaguchi (1968) found urocanic acid as a histidine metabolite in liver extracts from common carp, *Cyprinus carpio* and mackerel *Scomber japonicus*. Urocanic acid was also found in perchloric extracts of muscle tissue from Atlantic mackerel *Scomber scombrus* by Mackie and Fernandez-Salguero (1977). The isomers of urocanic acid were not separately measured in these reports. It is not clear why we did not detect cis-urocanic acid. If cis-urocanic acid was produced, it may have been transported from the skin to other sites in the fish before the assay was conducted.

Studies with marsupials and mice have supported the idea that the formation of DNA pyrimidine dimers can cause UVB-induced immunosuppression (Applegate *et al.*, 1989; Kripke *et al.*, 1992). This concept does not rule out an interaction of cis-urocanic acid with DNA. Pyrimidine dimers have been measured in fathead minnow embryos and in the skin of platyfish after exposure to ultraviolet radiation (Applegate and Ley, 1988; Ahmed and Setlow, 1993). We expect pyrimidine dimers to occur in the skin of UVB-exposed rainbow trout and are currently conducting studies to investigate any relationships among urocanic acid, pyrimidine dimers, and immunosuppression.

The levels of UVB that rainbow trout were exposed to in our studies simulated ambient solar levels observed at mid-latitudes. For example, on June 21, 1993, we measured a total broad-band UVB irradiance of 427 $\mu W/cm^2$ at 38°49'50" N latitude and at an altitude of 271 m above sea level in Columbia, MO. Correspondingly, the highest irradiance of UVB in our study was 357 $\mu W/cm^2$. We observed the same degree of UVB-induced effects in fish at the high irradiance of 357 $\mu W/cm^2$ and at the low irradiance of 190 $\mu W/cm^2$. Thus, the threshold for these effects was at or below 190 $\mu W/cm^2$, well below the solar UVB irradiance of 427 $\mu W/cm^2$.

The results of our study suggest that increased levels of solar UVB resulting from further ozone depletion may increase the risk of sunburn and fungal infection in natural fish populations. However, the extent of risk to fish populations must be assessed in terms of the degree and duration of exposure that fish may receive in nature. Exposure to UVB is a function of optical clarity of the water column, degree of tropospheric attenuation of UVB from clouds, dust and pollutants, and the extent to which fish are shaded during solar exposure.

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Chapter 19

Chronic Inflammatory Lesions in Two Small Fish Species, Medaka *(Oryzias latipes)* and Guppy *(Poecilia reticulata)*, Used in Carcinogenesis Bioassays

S.R. Abner[1], C.L. Frazier[1], J.S. Scheibe[1], R.A. Krol[2], R.M. Overstreet[2], W.W. Walker[2] and W.E. Hawkins[2,3]

[1] Department of Biology
Southeast Missouri State University
Cape Girardeau, MO 63701

[2] Gulf Coast Research Laboratory
Ocean Springs, MS 39564
[3] To whom correspondence should be addressed

ABSTRACT

Chronic inflammatory lesions affect two laboratory-reared fish species, the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) that are used as carcinogen bioassay models. The present study investigated some aspects of the biology of the inflammatory lesions and their relationships to responses by the medaka and guppy to two chemical carcinogens. A total of 5093 fish specimens from bioassays with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 2-acetylaminofluorene (AAF) were examined histologically to determine the prevalence, tissue distribution and morphology of chronic inflammatory lesions, and their relationship to responses to carcinogen exposure. Whereas the medaka typically exhibited a diffuse inflammatory response usually in the kidney, the guppy displayed morphologically diverse nodular lesions usually in the liver. Giant cells occurred frequently in kidney lesions of the medaka but not in the guppy. The occurrence of acid fast bacteria (AFB) was examined by Ziehl-Neelsen (ZN) staining of representative specimens of the two species from the DMBA study. Intact AFB were demonstrated in lesions in the guppy and ZN-positive debris in the medaka. About 40% of the cases in the guppy were associated with AFB, presumably a naturally-occurring mycobacterial infection. For each guppy and medaka with an inflammatory lesion, a degree of severity was determined and regression analysis (RA) used to examine factors that most influenced severity ratings. RA indicated that severity was higher in guppies, in older specimens of both species, in specimens exhibiting diffuse inflammatory reactions, and in AFB-positive cases. The results suggested that the inflammatory lesions occurred independently of neoplastic responses.

INTRODUCTION

The application of small laboratory fish species such as the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) as carcinogenesis models requires understanding of both the non-neoplastic and neoplastic lesions in the species. The occurrence of chronic inflammatory or granulomatous lesions is a major concern. Such lesions appear to affect most laboratory cultures of medaka and guppies, especially older specimens. Because of disagreements regarding the classification of cells comprising the lesions in fish, it has been suggested that they be referred to as chronic inflammatory foci rather than granuloma, the traditional term (Noga *et al.*, 1989, 1990).

Chronic inflammatory lesions frequently, but not always, have an infectious etiology. For example, in some human cases, the occurrence of granuloma may represent a host response to a neoplasm (Hollingsworth *et al.*, 1993). Whether or not chronic inflammatory lesions modify carcinogenic responses in fish is not known. Chronic inflammatory lesions may contribute to morbidity or mortality in fish by disrupting normal physiological processes (Smith and Gill, 1988). Following induction of chronic inflammatory lesions by an infectious agent, the effect on the immune system with regard to previous or subsequent carcinogenic stimuli is not known. Furthermore, because the inflammatory lesions are proliferative, there is the potential for misdiagnosis as a neoplasm (Harshbarger, 1984).

Bacteria, parasites, and fungi have all been implicated in the development of chronic inflammatory lesions in fish (Balouet and Baudin Laurencin, 1986). Among the bacterial agents, mycobacteria appear to be the most common cause of the lesions, especially those that develop into granuloma. Mycobacterial infection in fish is sometimes referred to as fish tuberculosis (Parisot and Wood, 1960), and described as a chronic systemic inflammatory disease that can affect any organ system frequently in aquarium-reared fishes (Van Duijn, 1981; Giavenni *et al.*, 1980; Leibovitz, 1980; Dulin, 1979).

The primary purpose of this study was to determine from histologic specimens the prevalence of chronic inflammatory lesions in medaka and guppies from tests that examined the carcinogenicity of 7, 12-dimethylbenz[α]anthracene (DMBA) and 2-acetylaminofluorene (AAF). We wished to determine whether the occurrence of the inflammatory lesions was induced or enhanced by carcinogen exposure. Also, the presence of acid-fast organisms, presumably mycobacteria in the inflammatory lesions, was determined by Ziehl-Neelsen staining of guppy and medaka tissue sections from the DMBA study.

MATERIALS AND METHODS

Fish

Histologic sections of medaka and guppy from bioassays of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 2-acetylaminofluorene (AAF) were examined. Because the lesions are considered to have an infectious etiology related to culture practices, culture conditions are presented in detail. Test medaka ultimately came from brood stock originally purchased in 1984 from Carolina Biological, Burlington, NC. They were maintained at a 3:2 female:male ratio with a loading density of about 1 fish per L in 40 L or larger aquaria containing well water that had been aged for at least 24 hr. Brood tanks were held at 25-28°C and provided a daily light regime of 16 hr light and 8 hr

dark. Fish were fed dry food (Prime Flakes, Zeigler Bros., Inc. or Stress Flakes, Aquavet)) sparingly 3 times daily and provided live brine shrimp (*Artemia* sp.) nauplii once daily. Feces and other debris were removed from the aquaria twice each week, with a concomitant water change of at least 20%. Aeration and continual water filtration were provided by biological sponge filters. Eggs were collected by siphoning them from the tank bottom, teasing them away from a netted female, or removing them from the sponge filters following egg deposition by the female. Individual eggs were placed in hatching solution in 4 L aerated jars at a density not exceeding 1 egg per mL. Temperature was maintained at 24°C. The hatching solution contained 100 mg NaCl, 3 mg KCl, 4 mg $CaCl_2.2H_2O$, and 16.3 mg $MgSO_4.7H_2O$ per 100 mL glass distilled water. Dead or diseased embryos were discarded. As fry hatched (about 10 day following fertilization), they were transferred to finger bowls containing approximately 1400 mL water with an average of forty fry per bowl and provided ciliates for 3 day and nematodes for 6 or more day. Bowls were maintained in a 27°C water bath. Feces and debris were removed daily with a concomitant 50% water change. After 3 day, newly hatched brine shrimp nauplii were added as a food source, and after 6 to 10 day fry were fed dry food and brine shrimp. Fry at or about 6 day age were transferred to beakers for testing or at about 15 day to maintenance aquaria for providing future brood stock. Water quality, temperature, and manipulations in maintenance aquaria were as described for brood aquaria with the exception of a 12 hr:12 hr light:dark cycle.

Initial guppy brood stock were purchased from Aqua World, Inc., St. Louis, Missouri. Brood guppies were maintained in 120 L aquaria at a loading density of approximately 1 fish per 2 L and a 3:5 male:female ratio. Most parameters were similar to those used for medaka. Newborn fry were afforded refuge in a thick floating vegetative mat of hornwort. Brood rooms were exposed to natural sunlight and enhanced by an artificial 12 hr light:12 hr dark photoperiod. During periods of fry collection, the photoperiod was increased to 16 hr light:8 hr dark. Brood fish were fed dry food three times daily and live brine shrimp nauplii once daily. In collection aquaria, feeding and grassed brood areas were divided by plexiglas strips to prevent food from entering protective vegetated areas. Fry were collected by netting and maintained at a density of 50-80 fry in 80 L aquaria filled with water from the brood aquaria. Fry aquaria were maintained under feeding, lighting, filtration, and temperature regimes identical to those in brood aquaria.

DMBA study

Tests using medaka and guppy incorporated two control groups and three DMBA treatment groups as follows: (1) an untreated control group, (2) a carrier control group exposed to 0.5 mL/L dimethylformamide (DMF), (3) a group exposed to a 0.45 μm filtrate of DMBA without DMF carrier (low concentration-about 0.5 ppm), (4) a group exposed to a 0.45 μm filtrate of DMBA with DMF carrier (intermediate concentration-about 20 ppm), and (5) a group exposed to a glass-fiber filtrate of DMBA with DMF carrier (high dose-about 50 ppm) (Hawkins *et al.*, 1989, 1990). The low concentration was prepared by adding granular DMBA (Aldrich Chemical Company, Milwaukee, WI) to 3 L of well water to produce a nominal concentration of 10 parts per million (ppm). This suspension was stirred in the dark for 3 or 4 day, filtered through a glass-fiber filter, then filtered again through a 0.45-μm Millipore® filter. A 200-mL portion of this filtrate was used for chemical analysis and the remaining 2,800 mL for exposure of the fish. Exposure media for the intermediate and high concentrations were prepared from a stock of DMBA dissolved in DMF. In both treatments where DMBA was dissolved in DMF, 3 L of well water were added to produce a nominal concentration of 10 ppm-DMBA. The intermediate dose was then prepared by passing the aqueous

DMBA-DMF suspension through a glass-fiber filter, then through a 0.45-μm filter. The high dose was prepared by passing the DMBA-DMF suspension through a glass-fiber filter only.

Exposures were carried out in 1L glass beakers in a carcinogen glove box (Labconco®) under static conditions, in the dark, at 26±1°C. Tests began with at least 145 specimens per test group. Specimens were 6-10 day old at initial exposure. Initial exposures lasted 6 hr after which the fish were removed to noncontaminated water for 6 day and then exposed three additional times for 6 hr each at intervals of 7 day. Fish were not fed during the 6-hr exposure periods. Following the fourth exposure, fish were moved to static growout aquaria containing noncontaminated water maintained at 26±1°C. Fish were fed dry flake food supplemented daily with live brine shrimp nauplii. Samples for histopathology were taken at 24 and 36 week post-initial exposure. Moribund specimens were also processed for histological examination as they were identified.

AAF study

Medaka and guppy were exposed to AAF by two mechanisms: (1) a single or multiple pulse exposure or (2) prolonged static-renewal exposure (Exposure media containing AFF was made up as described, James *et al.*, 1993). Exposures were static and were conducted in the dark or subdued light. Treatment groups for the pulse exposures included: 1 x 6 hr, 1 x 12 hr, 2 x 12 hr, 3 x 12 hr, and 4 x 12 hr. A 1 week interval separated the multiple (2, 3, and 4 x) exposures. For the static renewal exposures, treatment groups of medaka and guppies were exposed continuously for 168 hr (7 day) with renewal of AAF and control solutions every 24 hr. Controls were included for each treatment group. Pulse exposures were conducted in one control aquarium and one AAF treatment aquarium. Medaka and guppies were exposed simultaneously in the same treatment aquarium with specimens for each treatment contained in separate mesh chambers. Concentrations of AAF in exposure media were determined by dichloromethane extraction of a sample of the filtered suspension, and gas chromatographic analysis of the extract with flame ionization detection.

Each treatment consisted of 300 specimens. Medaka were 6-day post hatch individuals. Guppies were less than or equal to 48 hr post-parturition. One large initial pool of medaka fry was used for all treatments whereas each guppy group came from a set of fry collected weekly. After exposure, specimens were rinsed three times in well water, counted and placed in grow-out aquaria and held until sampling for histopathology at 24, 36, or 52 week post initial exposure.

For the static renewal test, the AAF stock solution was prepared as described for the pulse exposure. Medaka and guppies were exposed simultaneously to AAF in two separate 4 L beakers. Two additional 4 L beakers maintained under conditions identical to the exposure systems but without AAF, served as controls. Total volume in each beaker was 3 L with daily replacement from a single AAF/well water preparation for exposed fish or well water-only preparations for controls. A time zero water sample was taken immediately after the mesh chamber containing fish was introduced into the exposure aquarium. A 24-hr sample was taken the following day before the mesh chamber was removed to a new beaker containing fresh solution (well water or AAF), at which time another time zero sample was taken. At the end of the 7-day exposure the fish were rinsed, counted and placed in grow-out aquaria. Concentrations of AAF in both types of exposures were about 1.0 mg/L.

Histopathological procedures

Whole fish specimens were narcotized in ice water or MS-222 (tricaine methanesulfonate), the abdominal cavities of larger specimens were slit open and whole fish placed into Lillie's fixative (10% formalin; 85% saturated aqueous solution of picric acid; 5% formic acid). Depending on the size, specimens were fixed for 24 hr to 1 week. Extended fixation times insured decalcification of the larger specimens. Specimens were then dehydrated in a graded series of ethanol, cleared in xylene substitute and embedded in paraffin. Specimens were embedded side down for the DMBA test and belly down for the AAF test. Sections were cut at 5 μm in two separate planes and stained with hematoxylin and eosin. Liver was the expected target organ in the original carcinogenesis studies but other tissues appearing in those planes of section were also routinely examined.

Histologic evaluation

Slides of whole fish specimens recorded as having chronic inflammatory lesions when initially screened for neoplastic lesions were re-evaluated histologically. The following organs and tissues, when present, were examined in each specimen: eye, gill, heart, liver, kidney, spleen, intestine, ovary, testis, and musculature. The organs affected and the morphology of the chronic inflammatory lesions were recorded. Also, the overall tissue damage imposed by the inflammatory lesions was assessed and assigned a severity rating on a scale of 1 (slight) to 4 (severe), based on the extent of dissemination (number of organs affected) and the amount of normal tissue displacement (number and diameter of inflammatory lesions within organs). A system for classifying and coding histological data similar to that proposed by Reimschuessel *et al.* (1992) was used for the histological evaluation and regression analysis components of this study.

Ziehl-Neelsen Staining

To determine if acid-fast bacteria (AFB) were associated with the inflammatory lesions, Ziehl-Neelsen (ZN) staining was done on specimens from the original DMBA study, each representing a plane of section between the two slides examined for the histologic evaluation in the present study. The major criterion for a positive classification was the presence of intact magenta colored, rod-shaped bacteria. Slides were initially screened on low power and if there was no evidence of magenta particles associated with the lesions, the specimen was considered negative. If magenta particles were seen on low power, the specimen was examined further under oil immersion (1000X). If only acid-fast debris was seen at 1000X and intact rods were not clearly visible, then the specimen was considered negative.

Chi-square test

Prevalence data were analyzed by chi-square to test for the independence of lesion occurrence among individual and between pooled test groups. Chi-square comparisons of prevalence data were intended to determine if chronic inflammatory lesions were induced by carcinogen exposure.

Regression analyses

A stepwise multiple regression analysis was used to identify variables which most influenced the severity rating in each of the two studies. The default stepwise procedure (PROC STEPWISE) of Statistical Analysis System (SAS, 1982) was used to generate the regression models. The variables

tested were species, sex, age, exposure (consisting of 5 variables in the DMBA study and 8 in the AAF study), and lesion morphology. Another variable, presence of acid-fast bacteria, was tested in the DMBA study. For lesion morphology to be included in the analyses, each specimen was placed in one of two categories: "exclusively nodular" or "otherwise." The "otherwise" category included any cases exhibiting a nonspecific inflammatory reaction in addition to or independent of nodular lesions. Nominal scale data were treated as indicator variables (Neter and Wasserman, 1974). Regression analysis was primarily intended to determine if severity ratings were higher with any particular exposure regimen although other variables were included.

RESULTS

Prevalence of chronic inflammatory lesions

Table 1 shows the prevalence of chronic inflammatory lesions in medaka and guppies from the DMBA study. Chi-square analyses revealed no significant differences among individual test groups (medaka χ^2= 7.490; guppy χ^2= 7.040, p <0.05) or between pooled control and treatment groups (medaka χ^2= 0.187; guppy χ^2= 2.028, p<0.05). However, there was a significantly lower prevalence of chronic inflammatory lesions in those groups that used DMF for both medaka (χ^2= 4.873, p <0.05) and guppy (χ^2= 5.149, p <0.05) when control and low dose groups which did not incorporate DMF were pooled and tested against pooled solvent (DMF) control, middle dose and high dose groups that incorporated DMF.

Table 1
Prevalence of chronic inflammatory lesions (CIL) in medaka and guppy exposed to 7, 12-dimethylbenz[*a*]anthracene (DMBA).

	Medaka		Guppy	
Test Group	No. fish examined	No. with CIL (%)	No. fish examined	No. with CIL (%)
Control	170	12 (7%)	139	16 (12%)
DMF control	167	8 (5%)	129	8 (6%)
Low DMBA dose	173	14 (8%)	131	11 (8%)
Middle DMBA dose	155	7 (5%)	133	8 (6%)
High DMBA dose	77	0 (0%)	124	4 (3%)

Table 2 shows the prevalence of chronic inflammatory lesions from the AAF study. Although there were significant differences for the medaka (X^2= 22.529, p <0.01) and guppy (X^2= 42.07, p< 0.01) in lesion prevalence among individual AAF test groups based on chi-square analyses, no outstanding pattern was identified. There was a significantly higher prevalence of lesions in the pooled controls compared to the pooled treatment groups for the medaka (X^2=9.395, p< 0.01) only.

Table 2
Prevalence of chronic inflammatory lesions (CIL) in medaka and guppy exposed to acetylaminofluorene (AAF).

	Medaka		Guppy	
Test Group	No. fish examined	No. with CIL (%)	No. fish examined	No. with CIL (%)
Control/ 12 hr x 4	226	7 (3%)	252	22 (8%)
Control/ Constant	254	20 (8%)	235	59 (25%)
AAF 6 hr x 1	258	3 (1%)	255	74 (29%)
AAF 12 hr x 1	222	10 (5%)	273	55 (20%)
AAF 12 hr x 2	230	5 (2%)	260	39 (15%)
AAF 12 hr x 3	33	0 (0%)	232	49 (21%)
AAF 12 hr x 4	229	5 (2%)	239	29 (12%)
AAF Constant	238	8 (3%)	259	45 (17%)

Histologic Evaluation

Approximately two-thirds of the DMBA-exposed medaka, usually moribund specimens sacrificed prior to a scheduled sampling date, exhibited a nonspecific inflammatory reaction, predominantly in the kidney (Figure 1). Hematopoietic tissue of the kidney was often replaced by inflammatory cells, sometimes to the extent that few urinary tubules remained. Giant cells were frequently present within the extensive kidney lesions (Figure 2). In specimens from the AAF study, the kidney was rarely observed because of the belly-down embedding protocol used. However, when the kidney was present, the inflammatory condition was usually present although giant cells were less apparent. Nodular lesions were rare in the medaka.

The guppy lesions were mainly nodular ones in various stages of development (Figure 3) and exhibiting considerable morphological diversity (Figure 4). The liver was the organ most often affected. Staining characteristics of the early, solid inflammatory lesions ranged from pale basophilic to light brown. The necrotic, nodular lesions of the guppy took on many forms that varied from round-to-ovoid to large and irregular in shape. Melanomacrophage centers with dark pigment deposits were frequently noted in guppies (Figure 5).

Ziehl-Neelsen Staining

No intact acid-fast bacteria (AFB) were observed in medaka specimens from the DMBA test although faintly stained acid-fast debris was sometimes present. Of 47 guppy specimens stained by the Ziehl-Neelsen method, 19 (40%) were positive for AFB. Intact acid-fast bacilli were predominantly found in solid inflammatory lesions (Figure 6) but occurred also in necrotic lesions.

Figure 1. Medaka kidney with extensive inflammatory lesions. Control specimen, 57 week old. Hematoxylin and eosin.

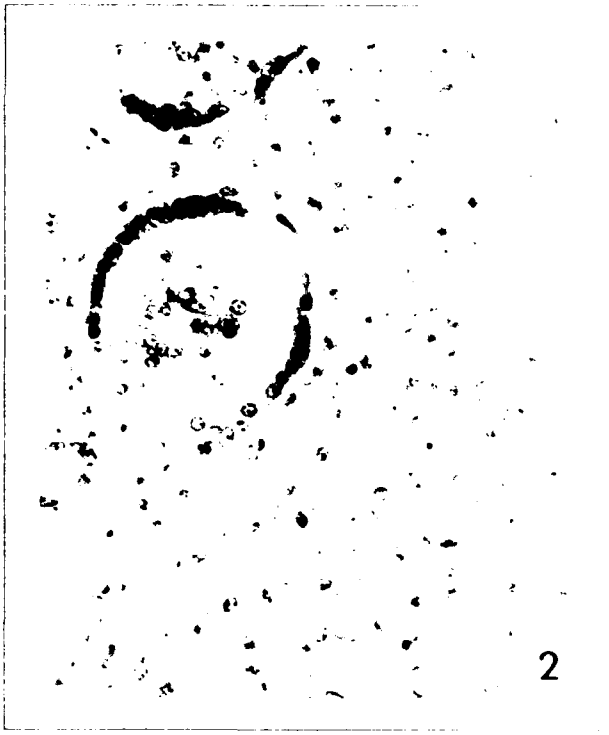

Figure 2. Multinuclear giant cell in kidney or medaka. DMBA exposed specimen, 55 week old. Hematoxylin and eosin.

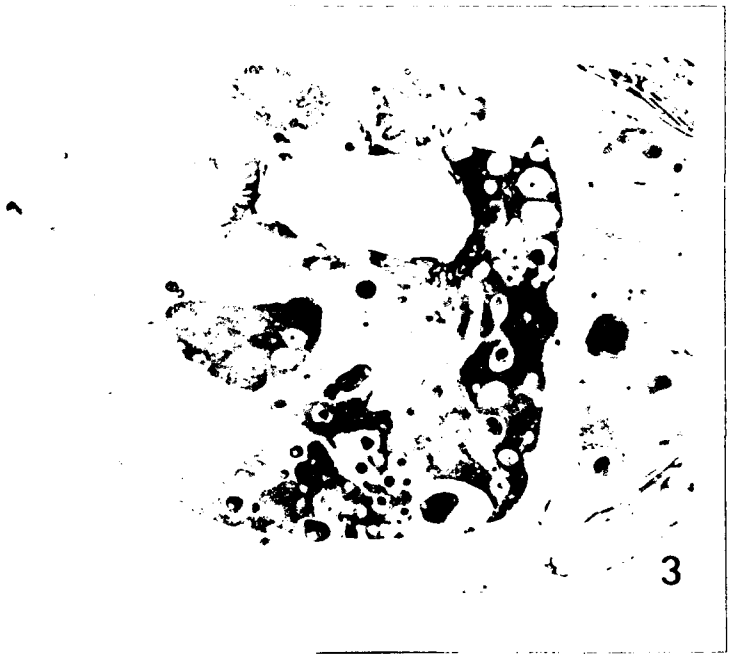

Figure 3. Widespread chronic inflammatory lesions in guppy. Note nodular lesions (from right to left) in cardiac region, liver, spleen and ovary. Specimen exposed to AFF, 29 week old. Hematoxylin and eosin.

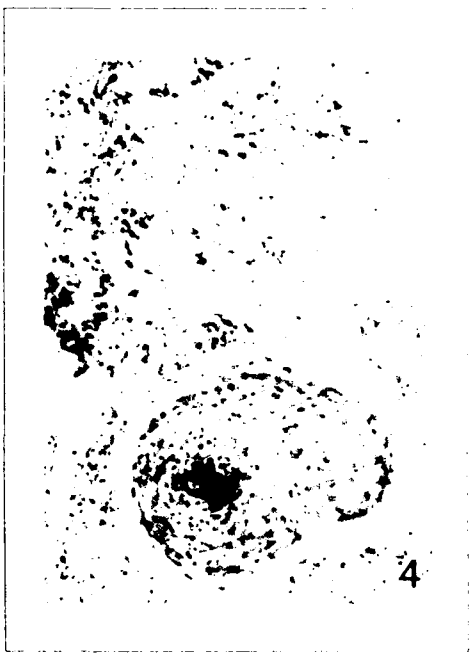

Figure 4. Diverse chronic inflammatory lesions in guppy kidney. Control specimen, 37 week old. Hematoxylin and eosin.

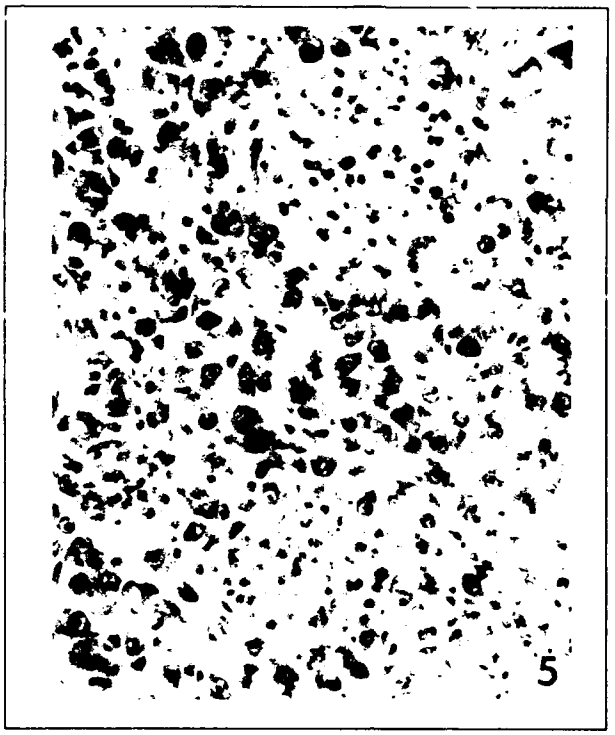

Figure 5. Two macrophage aggregates in the liver of a guppy. Specimen exposed to AAF, 52 weeks old. Hematoxylin and eosin.

Magnification:
Figure 1 = X70
Figure 2 = X480
Figure 3 = X15
Figure 4 = X200
Figure 5 = X480
Figure 6 = X480

Figure 6. Acid-fast structures (dark deposits), possibly bacteria, in a solid inflammatory lesion in the peritoneal cavity of a control guppy, 24 week old. Ziel-Neelsen stain.

Regression Analysis of DMBA Study

Lesion morphology, age, presence of AFB, and sex were entered into the regression model for the DMBA study (Table 3). Data for medaka and guppies were analyzed as one pooled group. The severity rating correlated positively with age, the presence of nonspecific inflammatory reactions, and the presence of AFB in the lesions. Also, males had significantly lower severity ratings.

Table 3
Stepwise regression analysis to examine factors most influencing the severity ratings of chronic inflammatory lesions in medaka and guppy exposed to DMBA

Model Statement: SR= SP SEX1 SEX2 AGE X1 X2 X3 X4 MORPH AFB

Parameter					
	df	**SS**	**MS**	**F**	**P**
Regression	4	42.97	10.74	19.09	0.0001
Error	78	43.89	0.56		
Total	82	86.87			
	Beta	**SE**	**SS**	**F**	**P**
Intercept	2.12				
MORPH	-1.00	0.19	15.19	26.99	0.0001
AGE	0.03	0.01	10.05	17.86	0.0001
AFB	0.81	0.22	7.89	14.03	0.0003
SEX1	-0.42	0.18	3.12	5.55	0.0210
R^2= 0.4947					

Abbreviations: SR= severity rating; SP= species; SEX1= males; SEX2=females; X1= solvent control; X2= low dose; X3= intermediate dose; X4= high dose; MORPH= lesion morphology; AFB= acid fast bacteria; df = degrees of freedom; SS = Type II sum of squares; MS = mean square; F = F statistic; P = probability; Beta = partial regression coefficient estimating β (beta); SE = standard error.

Regression Analysis for AAF Study

Lesion morphology, species, age, and four exposure variables (AAF 6 hr x1, 12 hr x2, 12 hr x3, 12 hr x4) were entered into the regression model for the AAF study (Data for medaka and guppy were analysed as one pooled group, Table 4). As in the DMBA study, severity was positively related to age and the presence of nonspecific inflammatory reactions. Guppies had significantly higher severity ratings than the medaka. The severity rating was positively related to the 6 hr x 1 exposure variable and negatively related to the 12 hr x 2, 12 hr x 3, and 12 hr x 4 exposure variables.

With the exception of the AAF 12 hr x 2 exposure variable, all variables included in the regression models were significant at the 0.05 level. The model for the DMBA study explained 49% of the variance, whereas the AAF model explained only 12% of the variance in the data set.

Table 4
Stepwise regression analysis to examine factors most influencing the severity ratings of chronic inflammatory lesions in medaka and guppy exposed to AAF.

Model Statement: SR= SP SEX1 SEX2 AGE X1 X2 X3 X4 X5 X6 X7 MORPH					
Parameter					
	df	SS	MS	F	P
Regression	7	48.84	6.98	8.21	0.0001
Error	422	358.41	0.85		
Total	429	407.25			
	Beta	SE	SS	F	P
Intercept	2.44				
MORPH	-0.74	0.13	25.26	30.45	0.0001
X5	-0.53	0.15	11.02	12.97	0.0004
X2	0.41	0.12	9.19	10.83	0.0011
SP	-0.44	0.14	8.58	10.10	0.0016
AGE	0.01	0.00	5.55	6.53	0.0109
X6	-0.35	0.17	3.63	4.28	0.0392
X4	-0.23	0.15	1.97	2.32	0.1283
R^2= 0.1199	Abbreviations: SR= severity rating; SP= species; SEX1= males; SEX2=females; X1= constant control; X2= 6hr x 1; X3= 12hr x 1; X4= 12hr x 2; X5= 12hr x 3; X6= 12hr x 4; X7= constant AAF; MORPH= lesion morphology				

DISCUSSION

For medaka and guppies in the DMBA study, the prevalence of chronic inflammatory lesions decreased with increasing DMBA concentration, possibly as an indirect result of DMBA toxicity. In many cases, moribund specimens in the high DMBA exposure groups were sacrificed prior to the scheduled sampling dates. Possibly this did not allow time for development of the inflammatory lesions. Supporting this view is the fact that mycobacteria have a relatively long generation time. Alternatively, the sparsity of inflammatory lesions in the guppy and their absence in medaka from the high dose DMBA exposure may have resulted from the chemical suppression of the inflammatory response. DMBA is known to possess immunotoxic properties (Dean and Murray, 1991; Ward *et al.*, 1985). Furthermore, chi-square analyses clearly indicated a significantly lower prevalence of inflammatory lesions in those groups exposed to DMF. Perhaps the DMBA and DMF acted together to repress activation of inflammatory cells. Another possibility is that the DMBA or DMF exerted anti-microbial effects inhibiting proliferation of mycobacteria in the hosts and, consequently, the associated inflammatory lesions were diminished.

In the AAF study, the prevalence of inflammatory lesions appeared to fluctuate randomLy among test groups with no apparent trends. However, chi-square analysis of the medaka AAF test showed a significantly lower prevalence in pooled treatment groups compared to pooled controls. Similarly, this finding suggests an inhibitory effect on the inflammatory response associated with AAF exposure.

Medaka and guppy exhibited distinctly different morphological inflammatory responses. Lesions in the medaka were primarily diffuse reactions in the kidney, often with giant cells, that were not associated with the presence of acid-fast bacteria. Based on reports that giant cells are not typically associated with mycobacteriosis in fish, the absence of intact acid-fast bacteria, and the lesion morphology being inconsistent with descriptions of mycobacterial lesions in the Lature (Wolke and Stroud, 1978), a noninfectious or infectious agent(s) other than mycobacteria could have been responsible for the chronic inflammatory reaction in the medaka.

The guppies typically exhibited discrete nodular lesions, frequently in the liver, and AFB were repeatedly detected. Based on demonstration of AFB in lesions as sufficient evidence for diagnosis as mycobacteriosis (Snieszko, 1978), and the lesion morphology being consistent with descriptions by Wolke and Stroud (1978), 40% of the cases in the guppy in the DMBA study can be attributed directly to a naturally occurring mycobacterial infection.

The regression models revealed significant differences in the severity rating with respect to age, lesion morphology, AFB, species, sex, and some of the AAF exposure variables. Severity ratings were consistently higher in older fish and in cases exhibiting nonspecific inflammatory reactions. Conceivably, the older the specimen, the more likely it is to have encountered granuloma-inducing agent(s) and the longer the proliferative, chronic inflammatory reaction has had to expand, spread, and displace more normal host tissue. The positive correlation of the severity rating with nonspecific inflammatory reactions is also easy to conceptualize, in that the lesion is not confined to a nodular focus. Its relatively diffuse nature would explain the associated higher severity ratings as more normal tissue is replaced. Similarly, with the presence of acid-fast bacteria there is the potential for spread of the organisms, resulting in widely disseminated chronic inflammatory lesions in an effort to contain the mycobacterial agent.

Guppies not only were more severely affected by the lesions than were medaka in the AAF study, but guppies also exhibited an equal or higher incidence of chronic inflammatory lesions than medaka in all test groups in the DMBA and AAF studies. Perhaps the medaka is less susceptible to the agents, including mycobacteria, responsible for the inflammatory lesions.

The reason for the lower severity ratings in male guppies from the DMBA study may be attributable to a venereal mode of transmission which predisposes the females to infection. At the time of fertilization, the gonopodium of the male enters the vent of the female delivering sperm which fertilizes the eggs. According to Anderson (1990), infectious agents in fish can be spread through the transfer of contaminated sex products. This is not an issue in the medaka whose eggs are externally fertilized. Although there was no evidence of chronic inflammatory lesions in developing guppy embryos, extensive granulomatous lesions were frequently present in the peritoneal cavity along the ovarian lining. If the infectious agent was present in the ambient water, contact between male and female guppies during sperm transfer could have resulted in the development of lesions in the peritoneal cavity in females, which would explain the lower severity ratings in males of the DMBA study.

In many of the AAF-exposed guppies, there were slight cases of exclusively solid, nodular macrophage aggregates that were considered to be early stage granulomatous lesions. Possibly some of these cases were actually melanomacrophage centers with little pigment deposition that were mistaken for early granulomatous lesions. Because these cases were typically assigned a rating of 1, an explanation is provided for the entry of the AAF exposure variables into the regression

model and their negative correlation with the severity rating. It is not surprising that the early, solid granulomatous lesions could be confused with melanomacrophage centers because of the ambiguous staining characteristics of hematoxylin and eosin. Melanomacrophage centers and granulomatous lesions are morphologically and functionally similar, as they are both discrete aggregates of macrophages that have a defensive role in sequestering antigens. Antigens and other particulates are deposited within melanomacrophage centers and, consequently, melanomacrophage centers may become foci for granuloma formation, especially in diseases such as mycobacteriosis where the antigen persists (Ferguson, 1989).

Exposure to DMBA and AAF was not responsible for the formation of inflammatory lesions based on the finding that there was an equally high or higher prevalence of inflammatory lesions in pooled controls when compared to pooled treatment groups in both studies. There was, however, evidence that all three chemical compounds (DMBA, AAF, and DMF) involved in this study exerted suppressive effects on the inflammatory responses of test species.

Inflammatory lesions in guppies were at least in part associated with acid-fast bacteria, presumed to be mycobacteria. Macrophages, which are traditionally believed to be the primary constituent of inflammatory lesions, play an important role in tumor surveillance and, according to Adams (1976), mycobacteria stimulate macrophages in such a way that neoplasia is inhibited and may even be reversed. Thus, an explanation is possibly provided for the fact that there were few cases of concomitant inflammatory lesions and neoplasms in this study. Perhaps those test specimens with inflammatory lesions possessed macrophages that were better able to ward off neoplastic cells.

In conclusion, the impact of a naturally-occurring mycobacterial infection on the immune system and the resultant influence on induction or suppression of chemically-induced neoplasia are not clear. In any case, attempts should be made to eliminate this infectious agent from test specimens that are to be used in carcinogenesis bioassays.

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Chapter 20

Polycyclic Aromatic Hydrocarbons Modulate the Macromolecular Synthesis in Hemocytes of the Eastern Oyster (*Crassostrea virginica*)

Mohamed Faisal[1] and Susanne Demmerle-Sami[2]

[1]Department of Environmental Sciences
School of Marine Science
Virginia Institute of Marine Science
The College of William and Mary
Gloucester Point, VA 23062

[2]Department de Biologie Moleculaire et Structurale
38041 Grenoble, Cedex, France

ABSTRACT

The effects of a polycyclic aromatic hydrocarbon (PAH) contaminated environment on macromolecular synthesis within hemocytes from resident *Crassostrea virginica* were analyzed using the uptake of radiolabelled precursors (thymidine, uridine, and leucine). The macromolecular synthesis was lower ($P<0.001$) in oysters from Hospital Point (HP, a PAH contaminated site) in the Elizabeth River (ER), Virginia, compared to oysters from the relatively clean Rappahannock River (RR). Maintaining the RR oysters at the HP site induced a statistically significant decrease in uridine uptake which is indicative of RNA synthesis. By depurating the HP oysters in the cleaner York River, a significant increase in RNA synthesis occurred over time as compared to the baseline values. This indicates that these changes are both inducible and reversible and, at least in part, are due to exposure to the ER sediment. Maintaining RR oysters in aquaria containing HP sediment inhibited uridine uptake by hemocytes.

Based on these studies, it appears that oyster hemocyte macromolecules are a target for PAH and its effects.

INTRODUCTION

Polycyclic Aromatic hydrocarbons (PAH) are ubiquitous contaminants of the marine environment. Once PAH is taken up by an organism and metabolically activated, it is believed that binding of PAH-reactive metabolites to the cellular macromolecules is the cause of disturbances in several

physiological processes at the cellular and molecular levels (Moore, 1985; Nishimoto and Varanasi, 1985).

The Elizabeth River, Virginia, is highly contaminated with creosote-derived PAH (Bieri *et al.*, 1986). Little is known, however, about the long term effects of residing in PAH contaminated environments on the marine benthic organisms. The decline in the *C. virginica* population due to infectious diseases (Haskin and Andrews, 1988; Andrews, 1988) that coincides with an increase in PAH levels in the Chesapeake Bay (Huggett *et al.*, 1987) has raised a question about the role PAH may play in compromising the oyster's host defense mechanism(s).

Oyster hemocytes are believed to play an important role in host defense mechanism(s) (Fisher, 1988; Auffret, 1988). Our previous results (Sami *et al.*, 1992; 1993) indicated that oysters collected from Hospital Point (HP), a site in the Elizabeth River where sediments are known to be contaminated with high levels of total PAH (Bieri *et al.*, 1986), are smaller in size and had fewer concanavalin A binding sites on the surface membrane, as compared to oysters collected from pristine sites in the Chesapeake Bay. These changes in hemocytes were attributed to the exposure of these oysters to PAH-contaminated sediments, since depuration of these oysters in cleaner waters reversed these changes. Also, healthy oysters exposed to HP-sediment induced similar changes.

Since the targets for many PAH are cellular macromolecules, the determination of the effects of exposure to PAH on hemocyte macromolecular synthesis may reveal some aspects of the mechanisms of PAH immunotoxicity in oysters. Techniques employing radiolabelled precursors have been widely utilized to study the *in vitro* synthesis of cellular macromolecules, such as ^{3}H-leucine for protein synthesizing activity (Seglen, 1976), ^{3}H-thymidine for DNA synthesis (Zakharova and Wallace, 1986) and ^{3}H-uridine for RNA synthesis (Shopsis, 1984). *In vitro*, oyster hemocytes are generally in a non-proliferative state. However, synthesis of important cellular macromolecules including DNA, was observed for up to two weeks postincubation (Sami *et al.*, 1991). The present study was designed to examine the influence of *in vivo* exposure to PAH-contaminated sediments on macromolecular synthesis under field and laboratory conditions.

MATERIALS AND METHODS

Oysters

Eastern oyster, *C. virginica*, was collected from the Rappahannock River (RR) at Bowler Rock, Virginia and divided into three groups. The first group (60 oysters) was examined immediately to determine the rate of macromolecular synthesis in circulating hemocytes of RR oysters. This would act as a baseline value. The second group (45 oysters) was placed into the relatively clean York River (YR) at Gloucester Point, Virginia, from which sub-samples were taken after four, eight, and 12 weeks (n=15). The third group (75 oysters) of the RR oysters was transplanted into the Elizabeth River (ER) at Hospital Point (HP), where high levels of PAH contamination have been reported (Bieri *et al.*, 1986). RR oysters transplanted at HP were sampled at four, eight, and 12 (n=25) weeks. In addition, 100 HP oysters were collected and divided into two groups. The first group (25 oysters) was examined immediately to establish a baseline value (ER-BL) for the Elizabeth River oysters. The second group (75 oysters) was kept in the York River and was sampled at four, eight, and 12 (n=25) weeks. Except for the presence of chemical contaminants, almost all other physicochemical characteristics were identical in the different sites.

Collection of hemolymph

Contamination of oysters was performed according to protocols described by Faisal and Hetrick (1993). Hemolymph was drawn from the adductor muscle sinus. A notch was drilled into the shell at approximately one third of the oyster length from the hinge, adjacent to the adductor muscle. The hemolymph (approximately 1-2 mL/oyster) was collected with a 27-gauge sterile syringe. Hemocytes were kept in polypropylene vials at 4°C until the time of the assay.

Macromolecular synthesis of hemocytes

Hemocytes were suspended in JLP-ODRP-1 medium (La Peyre *et al.*, 1993) at a density of $1x10^6$ cell/mL, and dispensed into 96-well plates (100 µL/well). The cells were then incubated at 27 °C in the presence of 5% CO_2 tension for up to 90 days in humidified chambers. Following a designated postincubation, each well was pulsed with 1 µCi (suspended in 100 µL of the medium) of one of the following: ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-leucine (specific activity 6.7 Ci/mL, DuPont, Wilmington, DE). Twelve replicate wells were made for each radiolabelled compound. Following 18-24 hr of incubation, the pulsed cells were harvested with an automatic cell harvester (PHD^R, Cambrigde Tech, Baltimore, MD) onto glass fiber filters. Radioactivity was assessed in a liquid scintillation counter using Beckman Ready-Safe as scintillant. Growth medium was changed every 30 days.

Viability of hemocytes was determined in 12 replicate wells and treated as mentioned above except that 100 µL of medium was added in place of the radiolabelled compound. Nonadherent cells were obtained by aspirating the medium and rinsing twice with sterile seawater. Each rinse was added to the aspirated medium containing cells. Adherent cells were removed by incubating each well with 100 µL of a 0.25% trypsin/0.02% EDTA solution for 10 min. After incubation, the solution was pipetted vigorously and added to the nonadherent cells. Cell suspensions were centrifuged at 100 x*g* for 5 min at 4°C and the number of viable cells was then determined using trypan blue exclusion stain.

The absence of microbial contaminants was determined microscopically. Stained preparations were made by adding 0.1 mL of the cell suspension to cytocentrifuge buckets. Following centrifugation (400 x*g*, 5 min), the slides were stained with Giemsa stain and examined morphologically using oil immersion light microscopy. Contaminated samples were excluded from the study.

Effects of exposing oysters *in vivo* to PAH-contaminated sediments

Studies were done to assess whether the results obtained from the field investigation could be reproduced, by exposing oysters to PAH contaminated sediments under laboratory conditions. The investigation involved the collection of sediments from HP, Bowler Rock of the RR, and from the YR at Gloucester Point, VA. The sediments were analyzed for the presence of PAH by standard procedures (Bieri *et al.*, 1986). Each sediment was thoroughly mixed using a fiberglass-lined cement mixer and then evenly spread on the floor of a designated glass aquarium (80 liter) to form a 5-10 cm thick layer. Oysters were maintained on a plastic grid just above the sediment surface. RR oysters were then maintained over the HP sediments for up to eleven weeks, while HP oysters were maintained over the YR sediment for the same time period. RR oyster and HP oyster control groups were kept in the YR and ER sediments, respectively. Hemocytes were collected after four, eight, and eleven weeks. Each sample group consisted of 25 oysters. Oysters were maintained in

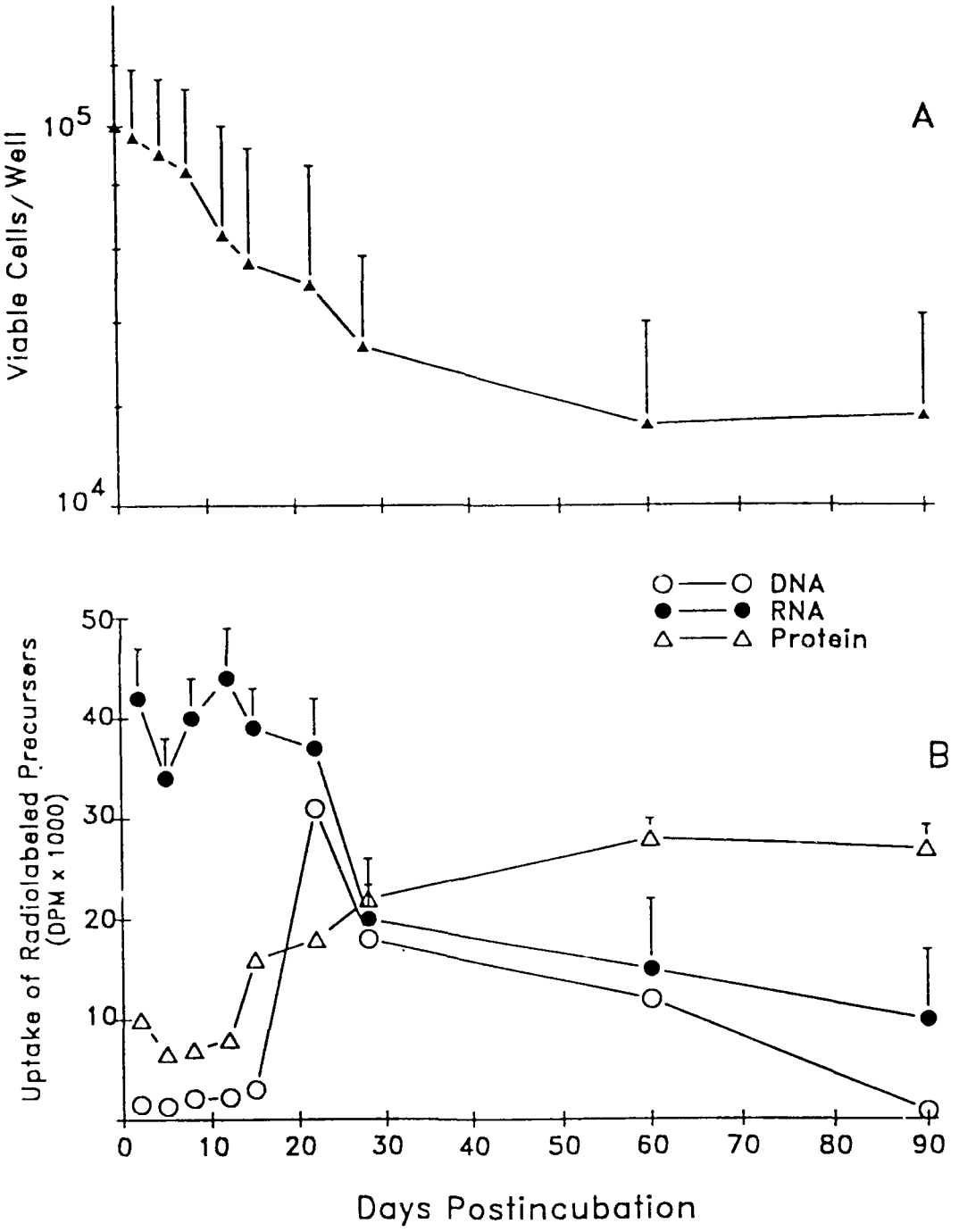

Figure 1. Viability **(A)** and macromolecular synthesis **(B)** in hemocytes of the Rappahannock Rivers oyster *in vitro*. Data are expressed as mean ± standard errors of disintegrations per minute.

a controlled static renewal system in a thermostatically controlled atmosphere (18 °C) and were fed with *Isochrysis galbana* algae.

Effects of *in vitro* exposure of hemocytes to PAH-contaminated sediments on uridine uptake

Sites in the Chesapeake Bay were selected based on their previous histories of contamination with PAH (C.L. Smith, Virginia Toxic Database, The Virginia Institute of Marine Science, Gloucester Point, Virginia). The Atlantic Wood site was chosen due to its record high contamination with PAH (2,200 ppm, Bieri *et al.*, 1986) and the fact that liver cancers were reported in resident fish (Vogelbein *et al.*, 1990). No oysters were present in the Atlantic Wood site. The York River site at Gloucester Point is included as a reference pristine site. Other sediments include HP, Craney Island and Water Side, all from the Elizabeth River.

Sediment samples were collected in the spring of 1990 from four sites: Atlantic Wood, HP, Craney Island, Water Side, and York River at Sarah Creek. Following extraction using standard methods (Bieri *et al.*, 1986), the organic residues were re-extracted with DMSO.

Hemocytes were collected and seeded in tissue culture 96-well plates as described previously, and were simultaneously exposed to a series of dilutions of the sediment extracts (six replicates/dilution). Following 24 hr of incubation, the cells were pulsed with ^{3}H-uridine (1 µCi/well) and assessed for radioactivity after 18-24 hr of additional incubation. The solvent control included hemocyte suspensions mixed with 100 µL of 0.001% DMSO in JLP-ODRP-1 medium.

Statistical analysis

Means and standard deviations were calculated. Variances from the group means were examined for uniformity using Bartlett's test and were then examined by analysis of variance (ANOVA). Significant differences were calculated using Dunnett's test. The significance level used was $P<0.05$.

RESULTS

Uptake of radiolabeled precursors

Uptake of each radiolabelled compound was followed in cultured hemocytes of Rappahannock River oysters for 90 days. On days 1, 4, 7, 11, 14, 21, 28, 59, and 89 postincubation, hemocytes were pulsed with the radiolabelled precursor and incubated for an additional 24 hr. The degree of incorporation of ^{3}H-uridine was 12-50 fold (42,700 DPM, $P<0.001$) higher than ^{3}H-thymidine (1,234 DPM) or ^{3}H-leucine (10,100 DPM) uptakes. As shown in Figure 1, a drop in the uptake of all radiolabelled precursors was apparent in the first week of postincubation, followed by a transient increase in the following two weeks. The number of viable hemocytes showed a steady drop with increasing postincubation times to 50% and 20% of original levels within 20 and 90 days postincubation, respectively. Despite the drop in cell numbers, macromolecular synthesis continued and protein synthesis increased ($P<0.001$; compared to the baseline value in hemocyte cultures (Figure 1).

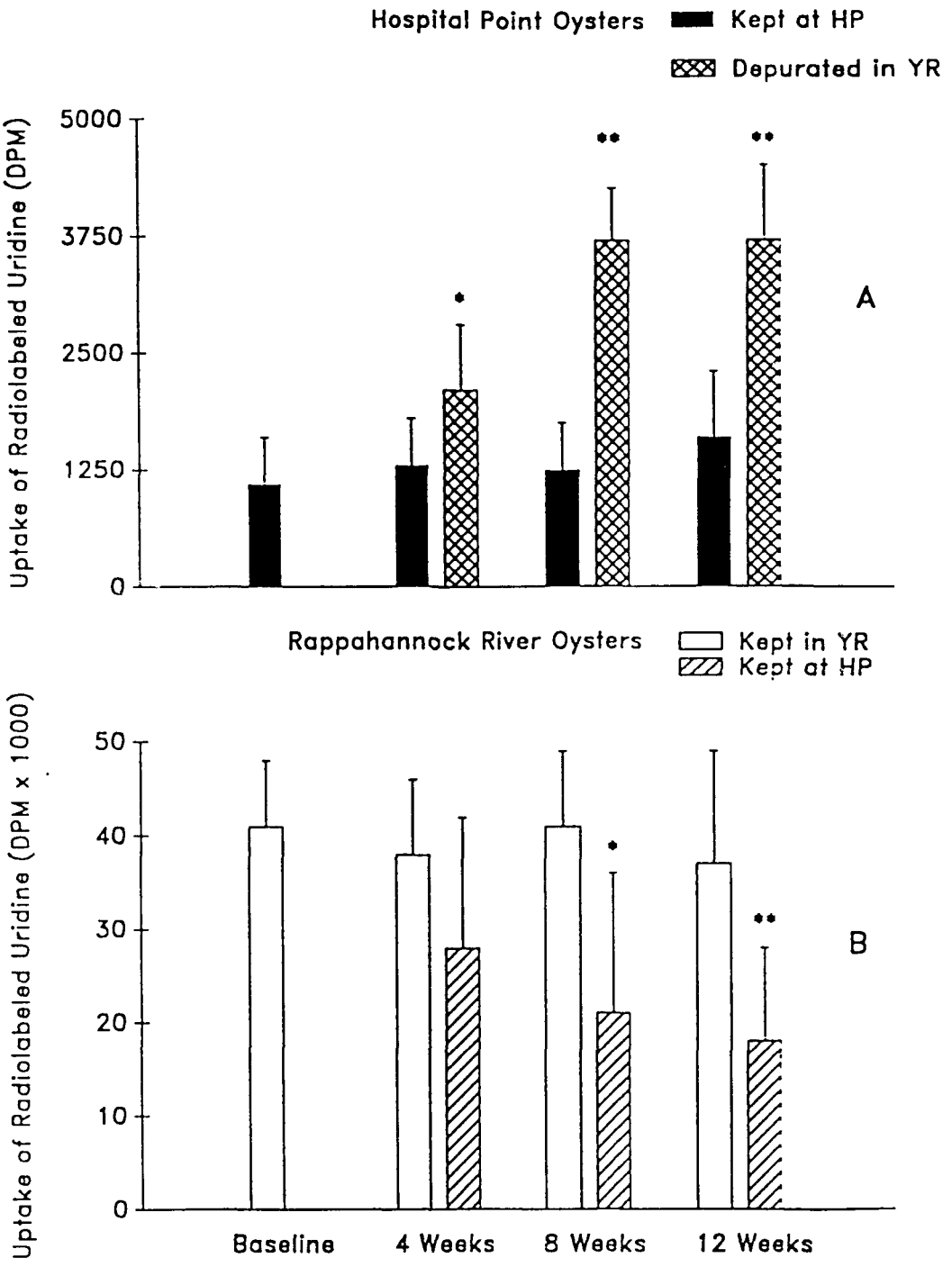

Figure 2. Uptake of tritiated uridine in hemocytes of oysters collected from Hospital Point (HP), a PAH-contaminated site in the Elizabeth River, and depurated in the York River **(A)**, and Rappahannock River oysters transplanted into the HP site **(B)**. Data are expressed as mean ± standard errors of disintegrations per minute.

No significant differences were observed in the baseline values (pulsed on day 0) by hemocytes between the YR and RR oysters. On the contrary, hemocytes of HP oysters had much lower levels (P) in the uptake of the three precursors (Table 1).

Table 1
Macromolecular synthesis of oyster hemocytes as measured by uptake of radiolabeled thymidine, uridine, and leucine. Data are expressed as means ± standard errors of disintegrations per minute (DPM).

Source of Oysters	^{3}H-Thymidine	^{3}H-Uridine	^{3}H-Leucine
Elizabeth River at Hospital Point	340± 67**	1,190± 310**	2,359± 421**
Rappahannock River at Bowler Point	1,234±310	42,700±9,675	10,100±3,605
York River at Gloucester Point	1,390±190^{NS}	39,410±8,910^{NS}	9,108±2,870^{NS}

^{NS}Not significant or ** significant at $P<0.001$ (compared to RR oysters)

Since the rate of uridine uptake was much higher than the uptake of thymidine or leucine, further experiments measured only the uridine uptake (pulsed on day 0).

Effects of Exposing Oysters to PAH-Contaminated Sediments Under Field Conditions

Hemocytes of ER oysters depurated in the YR showed a continuous increase in the rate of uridine uptake (P for 4 weeks and 0.001 for the 8 and 12 week samples, Figure 2A).

No significant difference occurred in the level of uridine uptake by hemocytes after maintaining RR oysters in the York River for 4, 8, or 12 weeks. On the contrary, keeping the RR oysters at the Hospital Point site for 8 or 12 weeks resulted in a significant reduction in the rate of uridine uptake ($P<0.01$ and 0.001 respectively) when compared to the RR oyster baseline values (Figure 2B).

Effects of Exposing Oysters to PAH-Contaminated Sediments in the Laboratory

To assess whether the results obtained from the field investigation could be reproduced, oysters were exposed to PAH contaminated sediments in glass aquaria.

Figure 3 shows the rate of uridine uptake in the RR oyster, maintained over ER sediment (20,000 ppb total PAH), decreased by 45, 53, and 90 % after 4, 8, and 11 weeks, respectively. RR oysters kept in the YR sediment maintained a relatively constant level of uridine uptake. In addition, uridine uptake by hemocytes of the ER oysters maintained over the YR sediment increased significantly, by 25, 70, and 100% after 4, 8, and 11 weeks of exposure, respectively. ER oysters kept over the ER sediments maintained very low rates of uridine uptake (Figure 3).

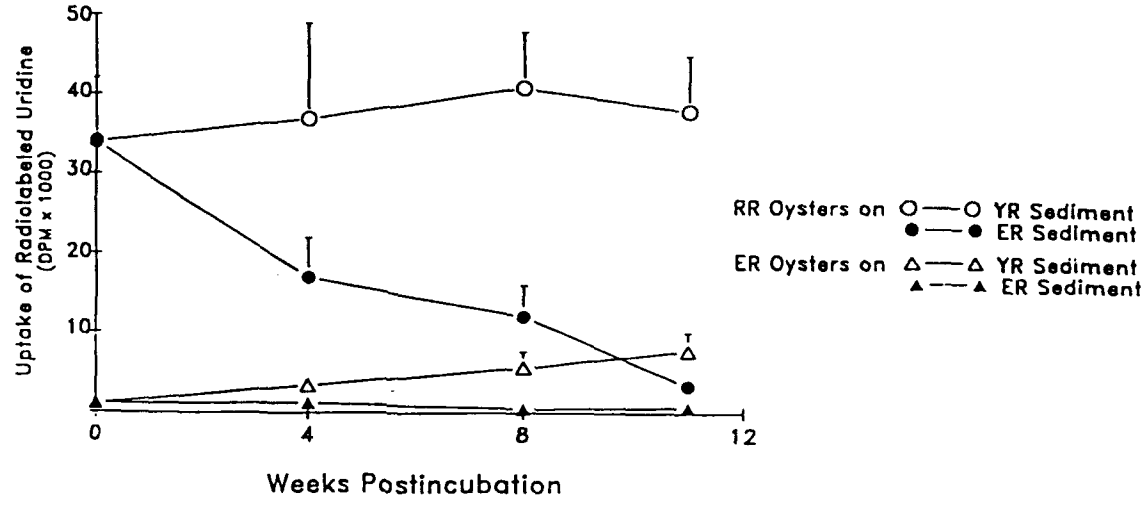

Figure 3. Effects of laboratory *in vivo* exposure to PAH-contaminated sediments on the uptake of tritiated uridine in oyster hemocytes. Data are expressed as mean ± standard errors of disintegrations per minute.

Exposure of Hemocytes *In vitro* to PAH-contaminated sediments

As shown in Figure 4, organic extracts of PAH-contaminated sediments modulated the level of uridine uptake by oyster hemocytes. This effect was, in some instances, independent of the PAH concentration. For example, extracts of Atlantic Wood sediment (2,200 ppm PAH) induced a significant stimulation in the level of uridine uptake as compared to DMSO-treated (control) cells. Only at a concentration of 25 µg sediment extract/mL was a significant suppression observed. On the contrary, sediments of the Water Side (17,000 ppb total PAH) and HP (20,000 ppb PAH) resulted in significant inhibition at a concentration of 10 µg extract/mL. Extracts from the York River (350 ppb PAH) and Craney Island (6,000 ppb PAH) caused no statistically significant changes when compared to the control.

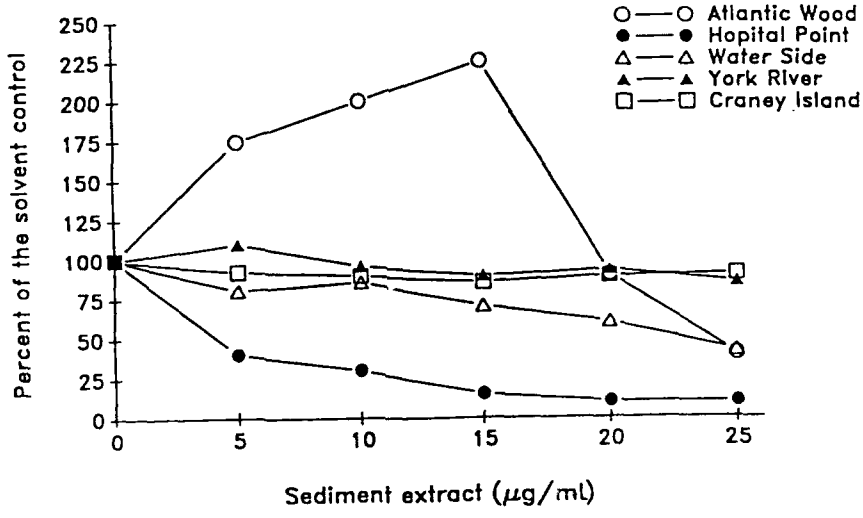

Figure 4. Effects of *in vitro* exposure to organic extracts of PAH-contaminated sediments on the uptake of tritiated uridine in oyster hemocytes. Data are expressed percentage of DPM given by DMSO-exposed hemocytes.

DISCUSSION

The results obtained from this study suggest that oyster hemocytes continue to synthesize cellular macromolecules under *in vitro* conditions for up to three months. Oyster cells maintained in culture without contamination for such an extended period of time is because of the development of a chemically defined medium that simulates the oyster hemolymph (La Peyre *et al.*, 1993) and a rigorous protocol for tissue decontamination (Faisal and Hetrick, 1993).

Of special interest was the high levels of RNA synthesis observed as compared to DNA and protein synthesis. The levels of DNA synthesis remained low with the continuous uptake of ^{3}H-thymidine indicating a chronic low level of DNA nucleotide synthesis. The relatively high uptake of ^{3}H-uridine indicates an activation of RNA synthesis. This activation may be part of an adaptive process designed to maintain physiological cellular processes under *in vitro* conditions (Darnell *et al.*, 1990). The increased protein synthesis that occurred over a 90 day period indicates that oyster cells were in a continuously active state of metabolism.

Table 1 and Figure 2 demonstrates that the hemocytes of HP oysters have a suppressed rate of macromolecular synthesis. Comparing the macromolecular synthesis in cells originating from different tissues (digestive gland, mantle epithelium, hemocytes, and heart-kidney) of HP oysters as compared to its counterparts in the RR oysters indicated that suppression of the macromolecules occurred only in hemocytes (Faisal and Hetrick, 1993). This suppression may be attributed to one of two possibilities. First, as shown by Sami *et al.* (1992), hemocytes of HP oysters are smaller in size as compared to RR oysters. Second, oyster hemocytes may be a target for the toxic action of PAH. It is well documented that PAH are immunotoxic to mammals (reviewed in White, 1986) and fish (Faisal *et al.*, 1991 a;b). Indeed, leukocytes of humans (Okano *et al.*, 1979), rodents (Kawabata and White, 1989), and fish (Van Veld *et al.*, 1993) were capable of transforming high molecular weight PAH compounds into reactive metabolites that then covalently bind to proteins and nucleic acids (Blondin and Viau, 1992; Mustonen and Hemminki, 1992). The cytochrome P450-monooxygenase or mixed function oxidase (MFO) system involved in the detoxification and transformation of PAH into reactive epoxides, dihydrodiols, quinones and phenolic derivatives has been identified in the digestive gland of several marine molluscs (reviewed in Buhler and Williams, 1989; Livingstone, 1985; Stegeman, 1981). Whether a xenobiotic biotransformation system similar to that found in vertebrate leukocytes, is also present in hemocytes remains to be elucidated.

The suppression in the rate of uridine uptake appears to be inducible, because RR oyster hemocytes showed a gradual decrease from the baseline values upon exposure to HP sediments under field and laboratory conditions. The condition of the HP oyster hemocytes also appears to be reversible because depurating ER oysters resulted in a gradual increase in the rate of uridine uptake. This indicates that these changes are, at least in part, due to exposure to the ER sediment.

Exposure of hemocytes to extracts of PAH-contaminated sediments *in vitro* simulated the results obtained *in vivo* (except for some concentrations of the Atlantic Wood sediments), *i.e.*, inhibition of uridine uptake. The degree of suppression, however, did not correlate with total PAH concentrations in these sediments. Furthermore, 5-15 µg of Atlantic Wood (AW) sediment extract induced more than 200% stimulation in the rate of uridine uptake. This is surprising since dilutions of AW sediment extracts had higher total PAH concentrations than in equivalent dilutions of extracts that induced suppression such as those from HP and Water Side sediments. This stimulation could be attributed to either the presence of some mutagenic PAH in the AW sediment that were less abundant

in the other sediment, or be a hermetic effect. Further analysis is being conducted to determine whether the suppression/stimulation obtained correlates with an individual or a mixture of PAH compounds.

The mechanism(s) by which exposure to PAH affects uridine uptake in oyster hemocytes warrants further investigation. Since phosphorylation of uridine by uridine kinase is the rate-limiting step in uridine uptake, toxicants that induce ATP depletion, or membrane damaging substances, can be expected to reduce the rate of uridine uptake rates (Shopsis, 1984). Several PAH compounds, such as phenanthrene, fluoranthene, pyrene, chrysene, benzofluoranthene, and benzo(a)pyrene, have been detected in Elizabeth River sediments (Bieri *et al.*, 1986) and are known to be toxic to cultured cells (Babish and Borenfreund, 1987).

Overall, the data obtained in this study, together with our previous investigations (Sami *et al.*, 1992; 1993) suggest that *in vivo* exposure to PAH-contaminated sediments induces not only functional and phenotypic alterations in oyster hemocytes, but also effects the synthesis of major cellular macromolecules, including nucleic acids. Despite the fact that these alterations are, at least partially, reversible, there is an increasing concern that the continuous influx of xenobiotics into the Chesapeake Bay may result in long term damage to the immunocompetence of Eastern oyster populations.

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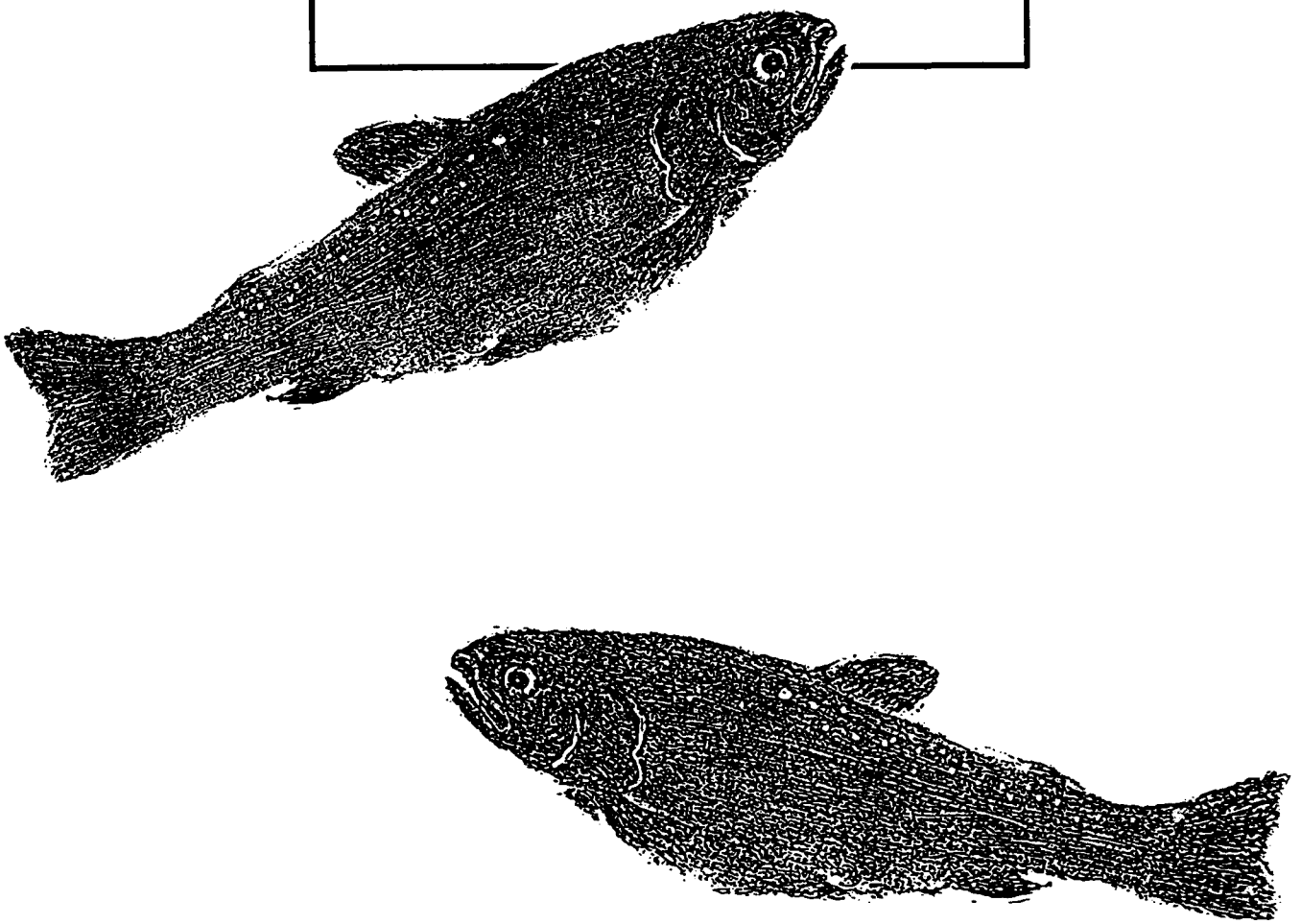

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